

# Phase I Final Report

June 1976

## Separation of Granulocytes from Whole Blood by Leukoadhesion

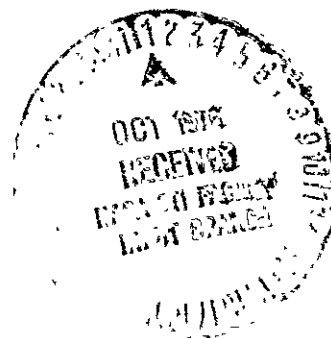
(NASA-CR-147883) SEPARATION OF GRANULOCYTES  
FROM WHOLE BLOOD BY LEUKOADHESION, PHASE 1  
Final Report (Martin Marietta Corp.) 64 p  
HC \$4.50

N76-31894

CSCL 06A

Unclas

G3/52 02486



**MARTIN MARIETTA**

MCR-76-272

PHASE I

NAS9-14545

FINAL REPORT

JUNE 1976

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SEPARATION OF GRANULOCYTES  
FROM WHOLE BLOOD  
BY LEUKOADHESION

Approved



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Prepared for

National Aeronautics and  
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## FOREWORD

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This document is submitted by Martin Marietta Aerospace, Denver Division, to the National Aeronautics and Space Administration, Lyndon B. Johnson Space Center in accordance with the reporting requirements of Contract NAS9-14545. The study was performed from April 1975 to June 1976 and was administered under the direction of Dr. Charles K. Lapinta, NASA-JSC Technical Monitor. Mr. Ralph N. Eberhardt, Aerothermal and Propulsion, was the Martin Marietta Program Manager. Mr. Paul E. Bingham of Martin Marietta assisted in the design, fabrication and test of subscale separator models.

Appreciation is extended to Dr. William A. Robinson and laboratory personnel at the University of Colorado Medical Center, Denver, Colorado, for the use of laboratory space and facilities, and technical assistance throughout the programs. Dr. Robinson is Associate Professor of Medicine, Head, Section of Oncology at the University of Colorado.

## ABSTRACT

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A technology program was conducted to investigate the use of capillary glass tubes for the separation and retrieval of large quantities of viable granulocytes and monocytes from whole blood on a continuous basis from a single donor. This effort represented Phase I, Feasibility Demonstration, of a three-phase program for development of a capillary tube cell separation device. The activity included the analysis and parametric laboratory testing with subscale models required to design a prototype device.

Capillary tubes 40 cm (15.7 in.) long with a nominal 0.030 cm (0.012 in.) internal diameter yielded the highest total process efficiency. Recovery efficiencies as high as 89% of the adhering cell population were obtained. Granulocyte phagocytosis of latex particles indicated approximately 90% viability. Monocytes recovered from the separation column retained their capability to stimulate human bone marrow colony growth, as demonstrated in an in-vitro cell culture assay.



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## I. INTRODUCTION

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Supplying large quantities of granulocytes for the investigation and treatment of leukemia and other blood disorders has received considerable attention in the past few years. The relatively short survival time of granulocytes in the circulation and the large daily turnover rate present a problem of procuring and storing sufficient cells for patient treatment. The production rate of granulocytes in the bone marrow has been estimated in the range of  $1.6 \times 10^9$  cells/kg/day. Assuming for ease of calculation a normal white blood cell count of 5000, it would take approximately 25 liters of whole blood to obtain sufficient white blood cells to replace normal daily loss (Ref. 1). Obtaining this quantity of blood from many donors places a severe demand on blood banks to process the blood and supply the quantity of cells needed for a single treatment. In addition, the use of many donors increases the possibility of receiving serum hepatitis and compounds the problems of transfusion reactions.

The desire to obtain large quantities of white cells in a more efficient manner while removing some of the risks associated with the collection led to the development of several continuous-flow separation devices. These leukopheresis devices were attached to a single donor whose blood was circulated through the separation apparatus over a 3-4 hour period. In this manner, a single donor could be used to obtain the same quantity of cells requiring multiple donors using the conventional techniques. The donor could be closely cross-matched with the patient (preferably a blood relative), decreasing the transfusion risk to the patient.

A review of the existing white blood cell separation techniques indicated, however, that there are problems in recovering large quantities of viable granulocytes with these devices. These techniques have proved somewhat inadequate either in terms of efficiency of separation or in preserving the viability of the collected cells. This led to the consideration of a unique device for separation and retrieval of granulocytes involving the use of capillary glass tubes. A brief discussion of the performance of existing separators and a summary of the capillary tube separation concept are presented in the following section. The Study Approach followed in conducting the feasibility investigation of the capillary tube concept is outlined in Section B.

## A. BACKGROUND

The development of the continuous flow centrifuge by IBM (Ref. 2), in conjunction with the National Cancer Institute, made possible the separation and collection of leukocytes from normal donors on an uninterrupted flow basis. With this machine it was possible to subject whole blood to a constant force field while collections of the desired components was in progress. The flowrate of the blood as it entered and traversed the centrifuge determined the length of time that the centrifugal forces acted and the depth of the film from which cells were collected. Selectively retrieving granulocytes from this film, however, was quite tedious and inefficient. Efforts to improve the efficiency of this type of device have met with mixed success to date. Granulocyte collection by these differential-centrifugation devices has resulted in average yields of  $4 \times 10^9$  granulocytes per 4-hour collection period, or roughly 1/25th of the amount needed to replace minimum daily losses (Ref. 3).

These low yields prompted the investigation of leukoadhesion as a means of granulocyte harvesting. Reversible absorption of granulocytes to nylon-wool filters (Leukopac, Fenwal Laboratories) was investigated by Djerassi using a procedure termed filtration leukopheresis (Ref. 4). Processing was relatively simple, as compared to differential centrifugation. Attempts to use this device and the method of Djerassi resulted in variable granulocyte recovery, and when granulocytes collected by the Leukopac were transfused, the post transfusion increments were significantly less than those observed with granulocytes collected by differential centrifugation (Ref. 5).

Because of these difficulties, a subsequent study was initiated (Ref. 6) in order to define more clearly the factors governing the binding and release of granulocytes by nylon-wool columns. Results indicated that granulocytes collected in this manner had nearly normal phagocytic and bactericidal function in-vitro. However, on transfusion, the granulocytes had a markedly shortened intravascular survival as compared to cells obtained by continuous-flow centrifugation. Apparently, the process of adhering to the nylon-wool altered the granulocyte surface in a way that lead to splenic uptake. In addition, significant platelet losses occurred using the filter. Vacuolization, fine structural defects and surface irregularities have also been noted in the cells, and functional deficiencies have been found (Ref. 7). A recent study investigating the functional abnormalities of human neutrophils collected with these filters showed that the extent of the abnormality is related to the time and degree of adherence to the nylon fibers (Ref. 8).

Difficulties with these techniques prompted Mr. Ralph N. Eberhardt of Martin Marietta Corporation, with consultation by Dr. William A. Robinson of the University of Colorado Medical Center, to investigate other approaches for collecting increased yields of viable granulocytes. A capillary cell separation technique was selected which utilizes the adhesive properties of the granulocytes (and monocytes) as the basis for separation. The device contains capillary glass tubes as the adhesive surface. The flow of blood through the small diameter tubes more nearly approaches the flow of blood through the body than does flow through a packed nylon-wool filter, such as the Leukopak. The non-adherent red cells, platelets and lymphocytes have a less tortuous path through the tubes and thus are subjected to a less severe environment before reinfusion into the donor. The small diameter tubes present a total surface area approximately two-thirds of that of beads of the same diameter, while still maintaining a high probability of granulocyte contact with inner and outer walls of the capillary tubes. The flow resistance through the capillary tube network remains constant with time when the device is used in a continuous flow loop with a donor. A nylon-wool filter, on the other hand, tends to pack at the low point in the flow apparatus making continual operation over a several hour period more difficult due to increased flow resistance. This increased flow resistance may be responsible for the loss of cell viability.

The design, fabrication and initial test evaluation of the capillary tube separator were explored under a Martin Marietta sponsored program conducted during 1971 (Ref. 9). Initial testing with the capillary cell separator indicated that removal of granulocytes could be accomplished by flowing whole blood through the column. Due to limited testing, the critical values of such parameters as capillary size (diameter and length), flowrate, operating temperature, and the composition and concentration of conditioning and washing solutions were not obtained. These parameters affect the overall efficiency of the process. A feasibility demonstration of this promising concept using a subscale separator model was then initiated as NASA-JSC Contract NAS9-14545. The study approach for this technology program is presented in the following section

## B. STUDY APPROACH

A flow chart summarizing the work to be accomplished is presented in Figure I-1. Task A, Program Definition, was a brief effort to prepare the overall plan to be followed during the remainder of the program. The cell separator is to be used to separate viable granulocytes from the whole blood of a donor on a continuous flow basis, with a minimum contamination of erythrocytes lymphocytes, and damaged cells, and to effect a minimal change to the remaining blood so that it can be returned to the donor. The technical approach for the cell separator is based on the high adhesiveness of granulocytes, as compared with erythrocytes and lymphocytes, towards certain surfaces, and on the ability to subsequently reduce this adhesiveness by chemical treatment to allow removal of the desired cells from the surface.

The following criteria were established as guidelines for the cell separator and directed the effort during this program:

- Any material used in the separator must be sterilizable and non-toxic to the cells,
- Any fluid used in the device must be compatible with blood and have the proper ingredients to preserve cell viability;
- The device should not remove more than 1 liter of blood from the donor at any time. The loss of red cells during the procedure should not exceed the quantity in one-half unit of blood (250 ml).
- The platelet count following this procedure, as measured in the peripheral blood, should not fall below 100,000/mm<sup>3</sup>.
- When this device is used to remove cells from a single donor, a maximum of four hours is anticipated for the procedure, and
- The quantity of viable granulocytes to be removed in a four-hour period is 10<sup>11</sup> cells.

The general operational procedure for the separator is as follows

- 1) To bring the adherent surface and the whole blood into contact under appropriate conditions and for a sufficient time to allow the surface to become coated with granulocytes,
- 2) To separate the remaining blood from the surface,

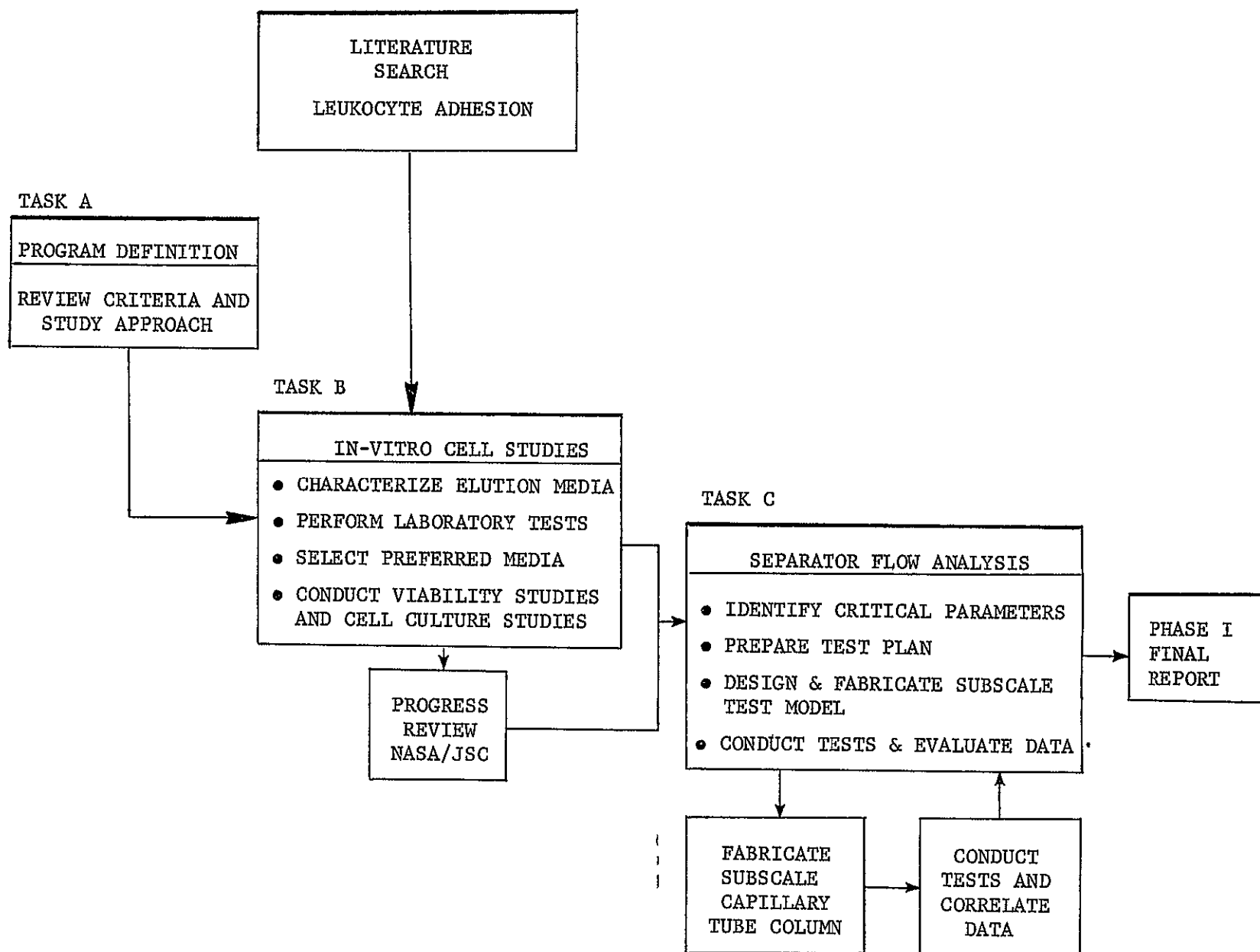


Figure I-1 Study Approach

- 3) To treat the adhering cells and surfaces chemically to reduce adhesion; and
- 4) To flush the cells from the surface.

During the study, each of the four phases of the process was to be investigated to identify the significant variables for each step and to quantitatively determine their effects. The evaluation of these processes was accomplished in two separate tasks, identified as Task B, In-vitro Cell Studies, and Task C, Separator Flow Analysis.

Task B involved in-vitro cell studies to analytically and experimentally evaluate various fluid media which tend to promote adhesion for cell separation, and which tend to reduce the adhesive properties of the cell membrane, initiating cell detachment from the wall. The type and conditioning of the attaching surface, the temperature of the surface and the reaction time for the processes to occur were considered

Task C considered the characterization of the flow processes occurring in a subscale column separator. The dynamics of the process were addressed; i.e., the effect of whole blood and fluid media flowrate on the attachment and removal of cells from the surface. A parallel plate flow model was fabricated and tested to optimize the diameter and length of the flow passage. A subscale capillary tube column was then fabricated and tested.

The Task B and Task C studies are discussed in the following chapters.



## II. IN-VITRO CELL STUDIES

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The Task B effort was both analytical and experimental in nature. The analytical portion was aimed at the characterization of various fluid media which tend to promote adhesion for cell separation, and which tend to reduce the adhesive properties of the cell membrane, initiating cell detachment from the wall. A discussion of the factors affecting leukoadhesion and their relationship to the capillary glass tube separator are presented in Section A of this chapter. Laboratory testing was performed to evaluate each of the factors important in the leukoadhesion process. A discussion of these experimental investigations is contained in Section B.

### A. FACTORS AFFECTING LEUKOADHESION

Important parameters affecting the magnitude of the force causing detachment between a cell and the surface to which it is adhering are the concentration of specific chemical additives, the type and conditioning of the attaching surface, the temperature of the surface and the reaction time for the process to occur. All fluid media must preserve the viability of all cells involved in the separation process.

As mentioned previously, the technical approach for the capillary cell separator is based on the high adhesiveness of granulocytes, as compared with erythrocytes and lymphocytes, towards certain surfaces, and on the ability to subsequently reduce this adhesiveness by chemical treatment to allow removal of the desired cells from the surface. The general operational procedure is as follows

- 1) To bring the adherent surface and the whole blood into contact under appropriate conditions and for a sufficient time to allow the surface to become coated with granulocytes,
- 2) To separate the remaining blood from the surface,
- 3) To treat the adhering cells and surface chemically to reduce adhesion, and
- 4) To flush the cells from the surface.

The goal is to provide viable granulocytes, with a minimum contamination of erythrocytes, lymphocytes, and damaged cells, and to effect a minimal change to the remaining blood so that it can be returned to the donor.

During the study each of the four phases of the process, as described above, was investigated in order to identify the significant variables for each step and to quantitatively determine their effects. A discussion of each phase of the process is presented below.

- 1) The forces that hold a cell to a surface are those of surface tension. Cells may fail to adhere because the angle of contact is nearly  $180^\circ$ , the cell is too rigid or spherical to provide sufficient surface contact, or the cell may be so fluid that it tears away from the surface even though a thin film of the cell is in contact with the surface. The forces of leukocyte-glass interaction have been extensively evaluated in-vitro by the use of a cover-slip chamber technique (Ref. 10-12). These studies suggest that contact interactions between cells and surfaces may be considered in terms of the balance of electrostatic repulsion and attractive interactions of the VanderWaals' type. It has been demonstrated that when strongly adherent cells are detached from glass, material is left behind on the glass surface due to nonlethal microruptures occurring within the cell periphery. This comes about because the total adhesive bonds between a cell and glass have greater energy than the total cohesive bonds holding the cell periphery together. Releasing the adherent cells with a minimum of change to the cell functional capability thus depends upon providing the right surface conditions to attach the cell with as weak a bond as possible so that when the cell is detached the cell periphery will be minimally affected.

The rate at which the adhering cells become attached to the surface depends mainly on (a) the nature of the surface, (b) the concentration of these cells in the adjacent fluid, (c) the fraction of surface not already covered by attached cells, (d) the flow field in the vicinity of the surface, (e) the chemical and cellular composition of the fluid, and (f) the temperature. Since whole human blood is to be used and returned to the donor, with at most the addition of an anticoagulant, (e) can be considered as fixed, and probably (f) also. Garvin (Ref. 13) has shown that adhesiveness is greatest in the range  $25^\circ$ - $44^\circ\text{C}$ . Above and below this temperature range the adhesiveness falls off, quite rapidly at the higher temperatures.

At the inlet to the separator, (b) will be fixed, but will otherwise depend on both time and location. Intuitively, it seems that the attachment rate is a linear function of both (b) and (c). The volume of cells applied is rather critical for efficient separation and granulocyte retention is somewhat dependent upon the number of granulocytes and platelets that are present (Ref. 14).

This leaves (a) and (d) as the major variables to be investigated for this portion of the process. As for the nature of the surface, granulocytes are less adherent to polished glass than to ground glass. However, the surface coating and the presence of certain fluid media are more significant to the attachment and viability of the cells. In some studies, the use of siliconized glassware was necessary, not only for the preservation of the leukocytes, but also to achieve a more complete removal of the red cells (Ref. 14). Cleaning of the glass surface is also important to preserving cell viability. Studies have shown (Ref. 14) that detergents should not be used for the recovery of leukocytes. Even prolonged washing and rinsing in water fails to remove the leukotoxic effect of the detergent. However, glassware that has been cleaned in acid and well rinsed appears to be safe for the handling of leukocytes. Item (d), the flow field in the vicinity of the surface, is a function of the Reynolds Number\* and the surface geometry. This item is the primary emphasis of Task C, where two different geometries, parallel flat plates and circular tubes, were investigated at a variety of Reynolds Numbers.

- 2) The separation of the remaining blood from the surface is accomplished by flushing with a fluid which meets the following requirements (a) since some mixing and diffusion with the blood will occur, it must be compatible, and (b) it should effectively remove the unwanted cells from the surface without disturbing the adherent cells. This latter property, which might be termed "washing efficiency," depends on, in addition to the nature of the fluid, the Reynolds Number, the surface geometry, and the temperature.

---

\* Reynolds Number is a dimensionless number that relates the fluid density  $\rho$ , velocity  $V$ , and viscosity  $\mu$  to a characteristic dimension  $D$  by the relationship  $Re = \frac{\rho V D}{\mu}$ .

- 3) It is desirable to release the adherent cells with a minimum of irreversible change to the cells. One approach to the release of adherent cells depends on the removal of  $\text{Ca}^{++}$  and/or  $\text{Mg}^{++}$  ions from the cells, which suggests the use of chelating agents. The relative importance of these two ions in cell adhesion has been evaluated by determining the adhesive characteristics of 5 day chick limb-bud cells in media containing various concentrations of these ions (Ref. 15). The cells were more adhesive in the presence of magnesium ions. When magnesium and calcium ions were present together at similar concentrations, the calcium ions modified the action of the magnesium ions. When the cells were plated out in a magnesium solution, the cells quickly spread and adhered to glass, while the cells that were plated out in a calcium containing solution remained rounded. A general conclusion from test data is that magnesium ions appear to be very important to cell adhesion while calcium ions appear to modify the strength of adhesion between the cells.

The lack of these ions in eluting solutions decreases the adhesive bonding capability of the cell peripheries, and the cells detach from each other and the attaching surface. This is a time dependent process which is sensitive to the temperature of the fluid media and attaching surface. Recent viability studies of cells obtained from dogs by the use of nylon filters suggested that in-vitro cell functions were enhanced when the cells were washed with 1mM magnesium chloride following release from the nylon fibers (Ref. 16). However, exposure of human neutrophils collected by filtration leukopheresis to calcium and/or magnesium cations did not alter their in-vitro functional defects (Ref. 8)

A literature search was made to review the results of studies investigating the effects of various eluting solutions on cells separated from whole blood by leukoadhesion. Rabinowitz (Ref. 14) found that granulocytes and monocytes attached to glass surfaces could be recovered with 0.2% EDTA in calcium and magnesium-free buffered saline. He also found that the presence of fresh serum was required for the adherence and retrieval of a high percentage of viable granulocytes. Most of the granulocytes were recovered in the first two-column volumes of effluent. Continued washing of the columns with the EDTA solution yielded a second peak of cells which consisted mainly of monocytes. Ninety-nine percent of the eluted cells excluded dye and 80 to 90% were capable of phagocytosis.

Herzig (Ref. 6) investigated the use of heparinized and ACD plasma in recovering adherent granulocytes from nylon-wool filters. He found that acidified heparinized plasma lacked chelating ability and that undiluted autologous ACD plasma yielded the greatest recovery of cells. The use of a 20% ACD plasma/saline mixture yielded comparable numbers of cells that demonstrated improved post-transfusion granulocyte increments over undiluted ACD plasma, although ACD plasma alone resulted in better in-vitro granulocyte function. The use of cold (4°C) eluting solution or elution under increased back pressure did not result in significant increases in granulocyte recovery.

An important operational and design consideration resulting from this study was that a linear relationship exists between granulocyte recovery and the extent of loading the attaching surface with adherent cells. In order to obtain a 50% recovery of cells,  $1.3 \times 10^{10}$  adhering granulocytes were required. However, the maximum recovery approached 75% of the adhering cell population, independent of loading of the surface.

Numerous evaluations have been made of granulocyte viability and function on cells harvested from nylon fibers using eluting solutions of the above mentioned type. Microscopic observations by Sanel (Ref. 17) revealed that granulocytes collected with an ACD plasma/saline mixture were about 20% visibly damaged. Vacuolization, clumping of intracellular granules and occasional nuclear extrusions were noted. The degree of damage ranged from barely perceptible to extreme. Wright (Ref. 8) reported that despite the structural defects and presence of apparently dead cells collected with ACD eluting solutions, viability of cells was greater than 90% by dye exclusion. Phagocytosis in some of the preparations of ACD-eluted cells appeared unimpaired despite cytoplasmic lesions. However, the cells appeared progressively more abnormal the greater the difficulty of eluting them from the surface. The variability in injury seems to depend on the amount of time a given cell is attached to the nylon fiber. Apparently the nylon interacts with the cell causing it to undergo a metabolic change that impairs cellular function and could lead to cell death.

Based upon the above information on cell adherence and detachment, several eluting solutions were formulated for evaluation during the in-vitro experimental investigations conducted as part of this task. These solutions included

- a) 20% heparinized plasma and 5% 0.1M EDTA in Dulbecco's Phosphate Buffered Saline (PBS),
  - b) 20% heparinized plasma in  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free HBSS,
  - c)  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ -free Dulbecco's PBS;
  - d) 20% EDTA (5mM) plasma in normal saline (0.9%),
  - e) 2mM EDTA,
  - f) 20% homologous ACD (Formula A) plasma in normal saline,
  - g) ACD (Formula A)/plasma/saline mixture.
- 4) The flushing of the released cells from the surface occurs in conjunction with step 3. Cell removal during step 3 depends on chemical effects at the interface of the cell and attaching surface. Hydrodynamic effects are also important in retrieving the cells from the separator column. These effects are measured during Task C for the candidate eluting fluids as a function of Reynolds Number, surface geometry, and temperature.

Resuspending the eluted cells in physiologic media appears to enhance their functional capacity. ACD eluted cells resuspended in balanced salt solutions, tissue culture media and heparinized plasma improved cellular morphology as determined by electron microscopy (Ref. 8). Cells in Hank's Balanced Salt Solution (HBSS) had fewer fissures and vacuoles, and all surfaces were less disrupted than the ACD cells. Cells washed and resuspended in tissue culture media or heparinized plasma had plasma membranes comparable to controls.

## B. EXPERIMENTAL INVESTIGATIONS

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Laboratory testing was performed to evaluate the parameters affecting the adherence and detachment of cells to a glass surface. The tests were performed using 100 mm diameter glass petri dishes (PYREX brand, Dow Corning) as the attaching surface. Normal human peripheral blood was collected in tissue culture Vacutainer tubes (No. 3208NT Becton, Dickinson and Company, Rutherford, N.J.) containing two drops of 1:1000 heparin (Liquaemin, Sodium "10", Organon Inc., W. Orange, N.J.). (Garvin (Ref. 13) has shown that heparin is the anticoagulant of choice when using leukoadhesion as the basis of separation of granulocytes and monocytes from other blood components.) Both whole blood and leukocyte rich plasma were used as the sources of cells for performing the studies.

Initial investigations concerned the type and conditioning of the attaching surface. Leukocyte rich plasma was used as the source of cells for this evaluation. Test plates were conditioned in the following manner.

- A) Acid washed, followed by rinsing 10 times with both tap water and distilled water.
- B) Acid washed, followed by 24-hour soak in 7X detergent, then rinsed 10 times with both tap water and distilled water.
- C) Acid washed, rinsed 10 times with both tap water and distilled water, and siliconized.
- D) Acid washed, soaked 24 hours in 7X detergent, rinsed 10 times with both tap water and distilled water, and then siliconized.

The siliconization was done with siliclad (Clay-Adams, Inc.) per a standard procedure used for glassware. The petri dishes were submerged in a 1% solution for 5 seconds, rinsed thoroughly, and baked in an oven for 10 minutes at 100°C. They were then sterilized.

An alternate to coating the glass surface to maintain cell viability is to wet the collecting surface with a priming media. For example, adhesion of leukocytes to glass has been found to be greater in alkaline solutions, and this stickiness was increased in hypotonic saline in the presence of plasma (Ref. 18). Thus, treating the glass surface with a 20% plasma/saline solution prior to applying the cells was also investigated.

A total of  $30 \times 10^6$  cells in 1.5 ml were applied to each plate. The plates were agitated for up to 15 minutes to allow the cells to come into contact with the glass surface. The cell suspensions contained approximately 90% granulocytes, the remaining cells being monocytes and lymphocytes. For this series of tests, Dulbecco's PBS Solution ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ -free) was used to evaluate recovery of cells from the plates. A summary of the pertinent test results is presented in Table I.

The siliconized surface, C (plate 3), yielded the greatest percentage of adherent cells. Condition D (plate 4) produced similar results (within the accuracy of the cell counting technique). A manual count was made using a hemocytometer. It, therefore, does not appear that washing the surface with 7X detergent prior to siliconization effects the adherence of the cells. Condition A (plate 1) resulted in a comparable number of adhering cells. However, microscopic observation of the recovered cells and the cells still remaining on the plate following application of a washing solution indicated that the cells were considerably more "roughed up" than for either C or D. Also, the cells still remaining on the plate appeared to be quite tightly attached, as indicated by their much greater spreading on the surface. The cells from plates 3 and 4 appeared to be much smaller, indicating that they tended to remain more spherical in shape, with much less of their cell membrane in contact with the glass surface. This is perhaps the reason why a greater recovery of the cells occurred for plates 3 and 4 than for plate 1. Results for plate 2 indicated that cells will not preferentially adhere to surfaces whose final treatment prior to water rinsing and sterilization is washing with 7X detergent.

Results for plate 5, as compared to plate 3, indicate that preconditioning the surface with a 20% plasma/saline solution definitely enhances the adhesion of cells to the surface. Also, the percentage of recovered cells is not as great if the glass surface is not preconditioned. The effect of incubation time of the wash solution on detachment and recovery of cells from the plate surface is indicated in Table I under plate 6. Plate 6 was conditioned exactly as plate 3. Approximately twice as many cells were recovered from the plate when the plate was incubated approximately twice as long at  $37^\circ\text{C}$  with Dulbecco's PBS solution.

A check was made of the viability of cells recovered from plates 3 and 6. A Trypan-blue dye-exclusion test indicated that 85-90% of the cells excluded dye. The capability of the recovered cells to phagocytize polystyrene latex particles, as an indication of their viability, was also evaluated. The latex particles were 1.099



TABLE I. EFFECT OF SURFACE CONDITIONING ON CELL ADHERENCE AND RETRIEVAL

TEST PLATE NO.	SURFACE CONDITIONING	20% PLASMA/ SALINE	NONADHERENT CELL COUNT	$\eta$ SEPARATION	RECOVERY INCUBATION TIME (37°C)	RECOVERED CELL COUNT	$\eta$ RECOVERY
1	A	Yes	$4.05 \times 10^6$	86.5%	2.0 Hr	$6.3 \times 10^6$	24.2%
2	B	Yes	$12.15 \times 10^6$	59.5%	2.0 Hr	$3.6 \times 10^6$	20.2%
3	C	Yes	$3.45 \times 10^6$	88.5%	1.5 Hr	$9.9 \times 10^6$	37.3%
4	D	Yes	$3.9 \times 10^6$	87.0%	1.5 Hr	$8.4 \times 10^6$	32.2%
5	C	No	$16.65 \times 10^6$	44.5%	1.5 Hr	$2.1 \times 10^6$	15.7%
6	C	Yes	$4.1 \times 10^6$	86.0%	3.5 Hr	$16.2 \times 10^6$	67.9%

- A. Acid washed, 10 rinses tap and distilled water.
- B. Acid washed, 24 hr. soak 7X detergent, 10 rinses tap and distilled water.
- C. Acid washed, 10 rinses tap and distilled water, siliconized.
- D. Acid washed, 24 hr. soak 7X detergent, 10 rinses tap and distilled water, siliconized.

microns in diameter and were suspended in HBSS. A mixture of particles and cells were incubated at 37°C, with shaking, for 30 minutes. A Giemsa-stained smear was made of the cells and approximately 90% of the cells appeared to contain particles.

Based upon these data, a procedure was established for preparation of all glassware, which included an acid rinse, siliconization of the surface and sterilization prior to testing. All glassware in subsequent testing was prepared by this procedure (e g , glass petri dishes, Pasteur pipettes, syringes, etc ). The only exception was the tubes in which the blood was collected. These tubes were pre-prepared and contained a vacuum. Some glass-adherent cells undoubtedly stuck to the walls of the tube. However, this phase of the study was only concerned with the viability of adherent cells on the prescribed attachment surface. Therefore, we were only concerned with the cells that were actually plated, and not the efficiency of removal of cells from the original sample.

Since data from Table I suggested that preconditioning the surface with a 20% plasma/saline mixture enhanced the adhesion of cells to the surface, a second series of tests was performed to further evaluate fluid media for preconditioning the attaching surface. A summary of typical test conditions and results is presented in Table II. The first four plates used blood from one donor, the second four plates used blood from a second donor. The differential white counts for the two donors were quite different, so differences are only significant within each group.

The first group of test plates indicated that preconditioning the surface with a 20% serum/Hanks' Balanced Salt Solution (HBSS) enhanced adhesion of cells to the surface, as indicated by the separation efficiency,  $\eta_{SEP}$ . The separation efficiency is defined as the number of adherent cells divided by the total number of white blood cells applied to the surface. The relatively low efficiency values are consistent with the presence of nonadherent lymphocytes in the original cell sample. The adherent cell population contained mononuclear cells (monocytes, and possibly some B-lymphocytes), as well as granulocytes. Examination of the nonadherent cell population indicated the presence of some granulocytes that apparently were not 'sticky' and therefore did not preferentially adhere. More of these nonadherent granulocytes were present when the plates were untreated, indicating that an active adherence factor may be present in the serum which is required for good adherence. This is consistent with data obtained by Rabinowitz (Ref. 14) for granulocyte adherence to glass beads.

TABLE II EFFECT OF FLUID MEDIA ON CELL ADHERENCE AND RETRIEVAL

TEST PLATE NO.	FLUID MEDIA CONDITIONING*	TEMPERATURE OF PLATE (°C)	ADHERENT CELL COUNT**	$\eta$ SEP (%)	WASH MEDIA FOR CELL RECOVERY	RECOVERY INCUBATION TIME (37°C)	RECOVERED CELL COUNT	$\eta$ RECOVERY (%)
1	Untreated	Room (23)	$31.1 \times 10^6$	50	0.002 M EDTA	2 Hr.	$0.6 \pm .3 \times 10^6$	2-3
2	Untreated	Room (23)	$37.6 \times 10^6$	60	↓	2 Hr.	$0.6 \pm .3 \times 10^6$	2-3
3	20% Se/HBSS	Room (23)	$39.9 \times 10^6$	64		2 Hr.	$0.6 \pm .3 \times 10^6$	2-3
4	20% Se/HBSS	Room (23)	$42.6 \times 10^6$	68		2 Hr.	$0.6 \pm .3 \times 10^6$	2-3
5	20% Se/HBSS	37	$18.0 \times 10^6$	24	20% Plasma, 5% .1M EDTA in Dulbecco's PBS ↓	5 Hr.	$16.6 \times 10^6$	92.2
6	20% Se/HBSS	37	$9.0 \times 10^6$	12		5 Hr.	$7.6 \times 10^6$	84.4
7	20% Plasma/ Saline	37	$11.9 \times 10^6$	16		5 Hr.	$12.0 \times 10^6$	99.9
8	20% Plasma/ Saline	37	$51.3 \times 10^6$	68		5 Hr.	$20.0 \times 10^6$	97.5

\* 20% Se/HBSS - 20% serum in Hanks' Balanced Salt Solution  
 20% Plasma/Saline - 20% Heparinized Plasma in Normal Saline (0.9%)

\*\* Adherent Cell Count = Total WBC applied to plate minus non-adherent cell count.

The second group of test plates was concerned with the use of plasma as opposed to serum as a preconditioning media. A lower total percentage of adherent cells from the total white blood cell population was obtained. The original cell sample was highly lymphocytic. However, the difference could not be explained merely by the decreased number of adherent cells in the original blood sample population. The possibility that the washing of nonadherent cells from the plate was so vigorous that it also mechanically removed some of the adherent cells suggested that a much less vigorous wash be used for plate 8. This resulted in a separation efficiency comparable to the first group. There did not appear to be a significant difference between serum and plasma adherence enhancement.

Several wash solutions were used to recover the adherent cell populations. The recovery efficiency is defined as the number of cells present in the eluting solution divided by the number of adherent cells on the glass surface. When 2mM EDTA was used by itself, very poor recovery was noted. However, when a mixture of 20% plasma and 5% 0.1M EDTA in Dulbecco's Phosphate Buffered Saline (PBS) was used, recoveries of the adherent cells averaged greater than 93%. This data, plus that from several additional tests using identical wash solutions, indicates that the presence of plasma is also important when detaching the cells from the surface.

A series of plates were tested to evaluate the relative recovery efficiencies of the eluting solutions itemized in Section A. The ACD plasma/saline solutions were not evaluated during this test due to availability; evaluation of these solutions was made during the capillary tube column test activity. The results are listed in Table III. Each of the siliconized plates was preconditioned with a 20% plasma/HBSS wash prior to exposing the cells to the glass surface. In each case, the wash solution was allowed to incubate on the plates at 37°C for three hours prior to collecting the cell-rich effluent. The 20% EDTA plasma/saline solution and the 20% plasma/5% EDTA/Dulbecco's PBS solution yielded comparable cell recovery efficiencies and were superior to the other solutions tested. Ninety-five percent of the granulocytes collected by these two solutions excluded dye and 90-92% were able to phagocytize latex particles.

Recent unpublished work by Dr. William A. Robinson's Laboratory at the University of Colorado Medical Center has shown that mononuclear cells, when plated as feeder layers in a cell culture system, will stimulate human bone marrow colony growth in Agar-gel. The data indicates that the monocyte is probably the source of stimulating activity, at least the stimulating activity is provided by an adherent cell population. This information suggested that the

viability of mononuclear adherent cells removed from a glass surface could be checked by such a cell culture technique. Since mononuclear cells are more difficult to remove from glass than granulocytes, a verification of their viability would indicate that the wash media might also preserve the viability of detached granulocytes. The cell culture technique used is that originally developed by Dr Robinson (Ref 19 and 20). A summary of the technique is included in the Appendix.

A relatively pure mononuclear cell fraction obtained by Bovine Serum Albumin (BSA) density gradient separation (Ref. 21) was applied to siliconized glass plates. Approximately 90% of the adherent cells were removed using a 20% EDTA (5mM) plasma solution in normal saline. The cells were plated at various concentrations with human bone marrow overlays. The number of colonies obtained at each of the concentrations plated is shown in Figure II-1. Blank feeder layers and blank overlays were used as controls and exhibited no colony growth. The amount of colony growth appeared to be typical of that obtained by using cells from the gradient that were not exposed to the glass surface, indicating good cell viability following removal from the glass surface.

TABLE III EFFECT OF ELUTING SOLUTION ON RECOVERY  
EFFICIENCY OF GLASS ADHERENT CELLS

<u>Eluting Solution</u>	<u>Recovery Efficiency</u>
20% Hep. Plasma, 5% 0.1M EDTA in Dulbecco's PBS	93%
2mM EDTA	2-3%
20% EDTA (5mM) Plasma in Saline	95%
20% Hep Plasma in $\text{Ca}^{++}$ and $\text{Mg}^{++}$ Free HBSS	72%
$\text{Ca}^{++}$ and $\text{Mg}^{++}$ Free Dulbecco's PBS	45%

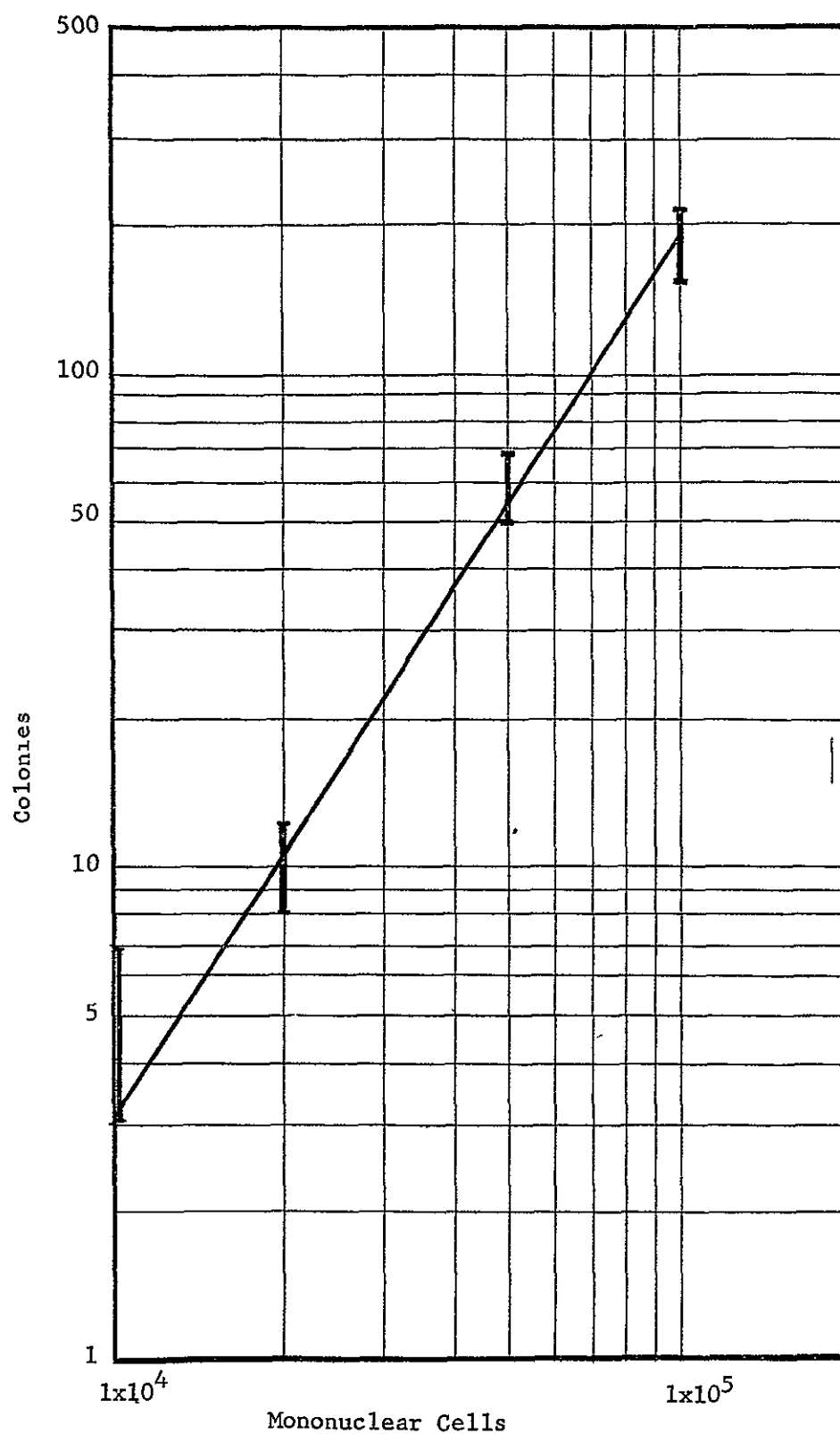


Figure II-1 Human Bone Marrow Colony Growth with Mononuclear Cells Eluted from Glass with 20% EDTA Plasma in Saline

### III. SEPARATOR FLOW ANALYSIS-----

The Task C effort was aimed at characterizing the flow processes occurring in a subscale column representative of a full-scale cell separator. The following factors were selected as important parameters to be evaluated under flow conditions:

- The composition and percentage of special fluid additives required at various stages of the flow process,
- The number, diameter and length of the capillary tubes,
- The flowrates of the whole blood, priming and washing solutions; and
- The temperature of the apparatus, and the blood and fluid media.

Test models for evaluating the flow processes were designed and fabricated. A simple parallel plate model was designed to obtain parametric test data. The model was made with two glass surfaces separated by a gasket-spacer and clamped together along the edges. The primary parameters to be established during the test were the flowrate, the spacing of the glass surface, and the distribution of cells attached along the length of the glass plate. A discussion of the parallel plate test apparatus and test results is contained in Section A.

Following the studies with the parallel plate apparatus and the evaluation of the test data, a subscale capillary column was fabricated. The dimensions of the capillary tubes (diameter and length) and the overall dimensions of the column were based upon the test data obtained from the parallel plate model. Separation and retrieval of granulocytes with the capillary tube model was compared with data obtained with the parallel plate model. The capillary tube subscale models and test results are presented in Section B.

#### A. PARALLEL PLATE MODEL

The parallel plate model was designed to conduct parametric testing for determining the best diameter and length of capillary tubes for a full scale separator design. The cell surface area-to-volume ratio was readily varied with this model by changing the spacing

between the glass surfaces. The model was designed to permit easy removal of the glass surfaces for microscopic examination of the adherent cell density and location as a function of fluid flowrate through the apparatus. The distribution of cells on the glass surface as a function of distance from a reference point was monitored by visual observation with a microscope. A motor driven syringe (Harvard Profusion Apparatus) was used to accurately control flowrate. A description of the flow model and test setup is contained below.

#### 1. Parallel Plate Model Description

A sketch of the model is shown in Figure III-1. It consisted of two flat glass plates 40 cm long, 5 cm wide and 0.6 cm thick. These plates were separated by variable thickness metal spacers, thus forming a narrow channel with parallel sides through which the blood flowed. Both the width and thickness of the flow channel were adjustable by the use of different spacers. The spacer-to-glass seal was accomplished by the use of teflon strips which also prevented blood from contacting the metal spacers. A glass plate assembly showing the teflon covered spacers is presented in Figure III-2.

A series of six small clamps along each side of the glass plate provided an inexpensive means of holding the plates and spacer assembly in position during testing. This also allowed the plates to be removed quickly following a test for staining and microscopic examination of the adherent cell distribution along the inner surfaces of the glass. Metal strips were used on the outer surfaces of the glass plates for anchoring the clamps. The assembled flow model is shown in Figure III-3.

Closure of the ends of the parallel plate model was provided by flow diffusers. These diffusers were fabricated from silicone rubber. They sealed against the outer rectangular cross section of the glass plates and terminated in a tubular cross section for connection into the flow system. The tube on each end was coaxial with the longitudinal axis of the model.

During checkout testing with the model it was found that these diffusers expanded under pressure. As a result of this expansion, these diffusers contained a large volume of blood as compared to the volume contained between the glass plates. The velocity of blood within the diffuser was therefore much lower than the velocity of blood within the flow channel. This resulted in some separation of the blood components prior to entering the flow channel because of red



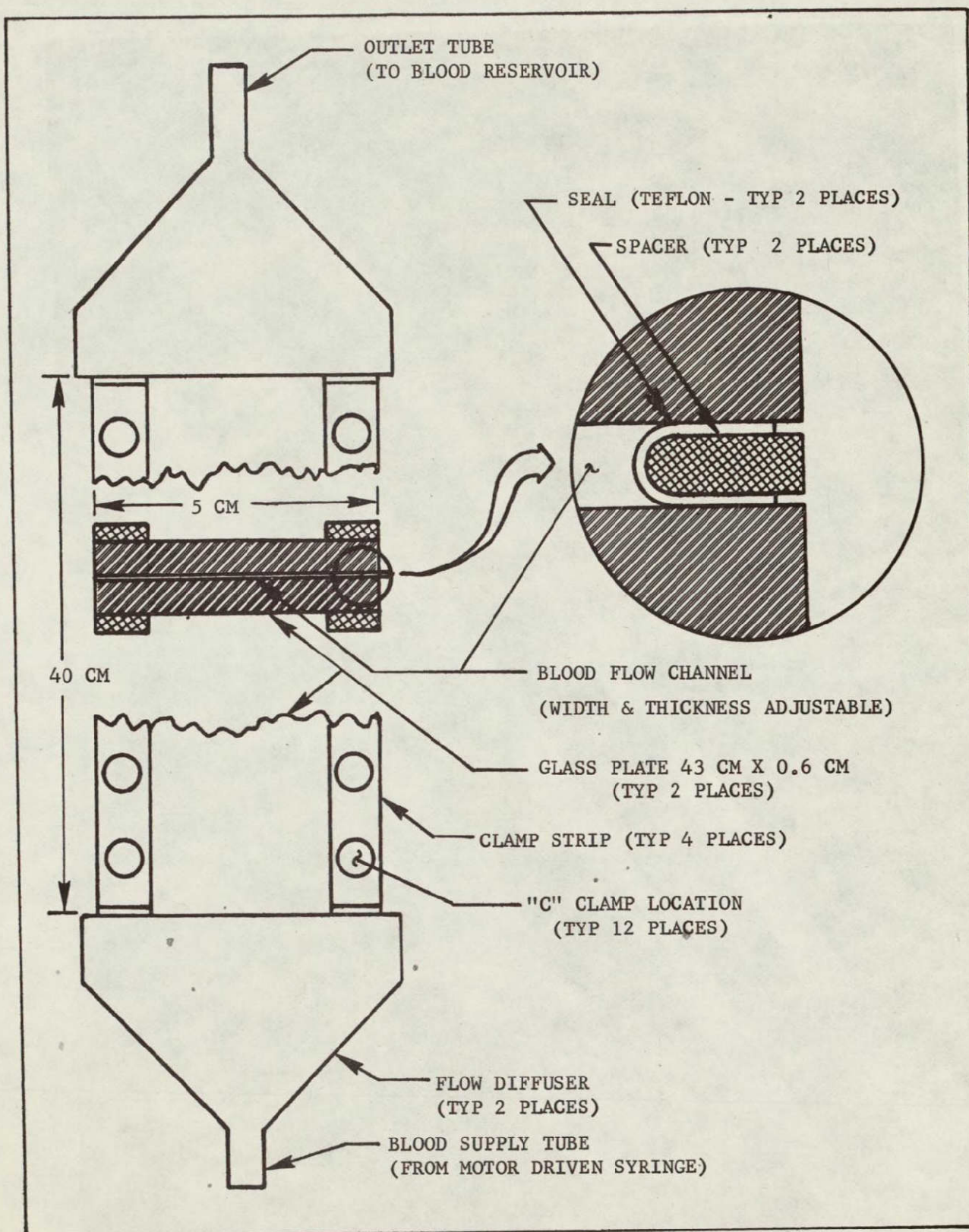


Figure III 1 Parallel Plate Flow Apparatus



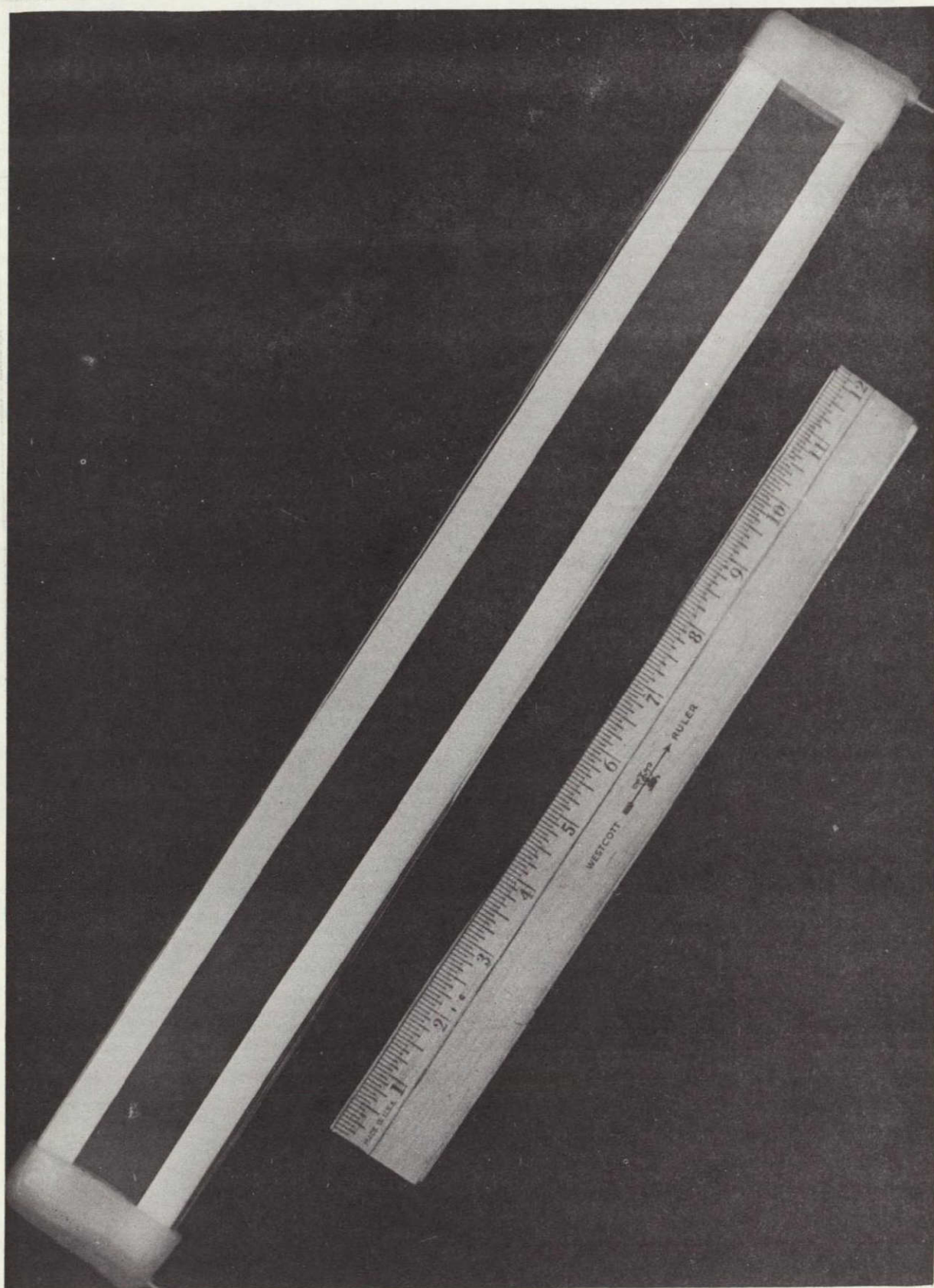


Figure III-2 Parallel Plate Model Showing  
the Teflon-Covered Spacers



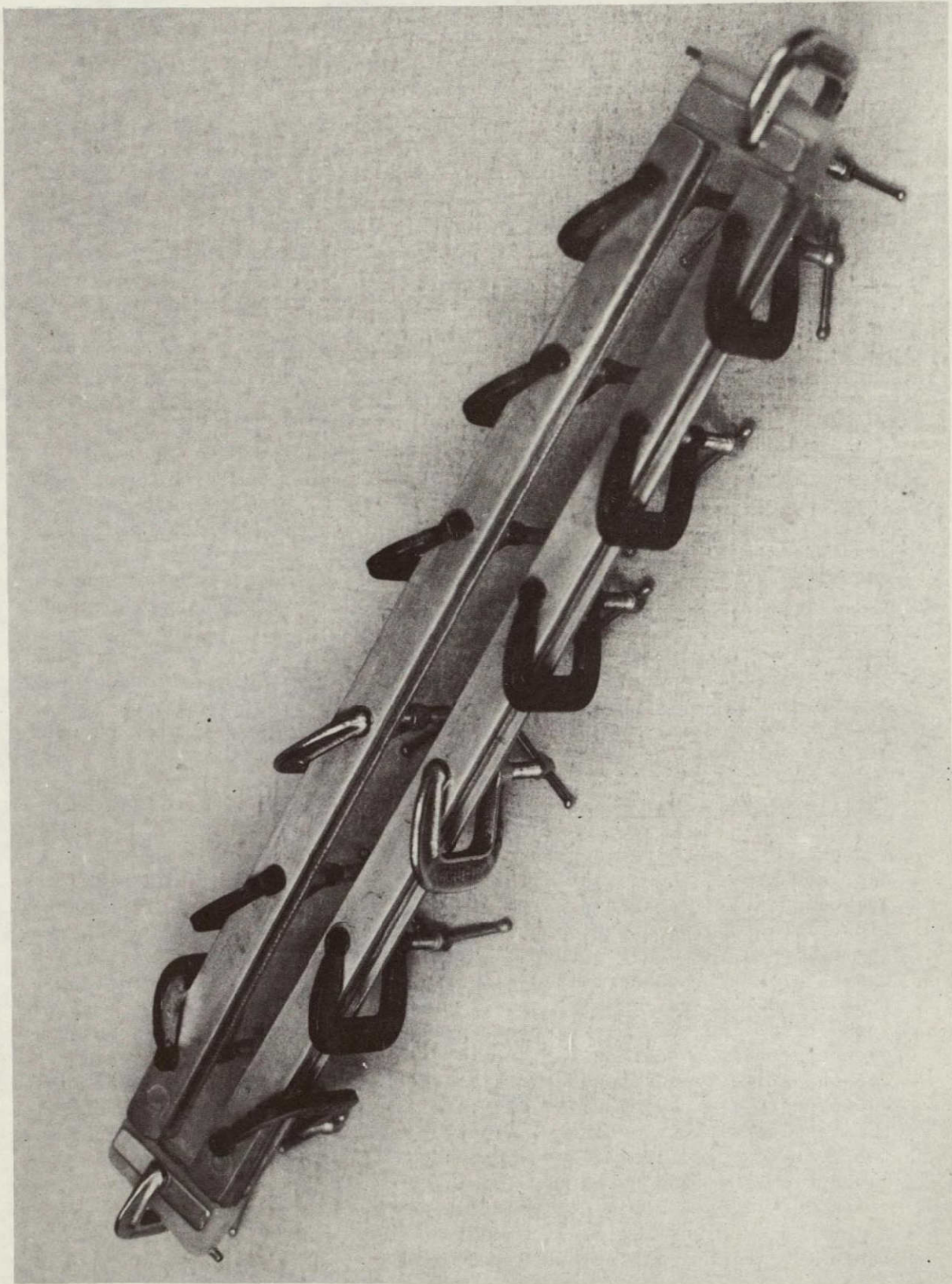


Figure III-3 Parallel Plate Model Assembly

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cell sedimentation. New flow diffusers were designed and fabricated to alleviate this problem. They were made so that flow entered and left the model perpendicular to the direction of flow through the column. With this approach an even distribution of flow across the width of the column was provided and the volume within the diffuser was reduced. Checkout runs indicated that a uniform velocity profile was maintained across the width of the flow section. The improved diffuser design is shown in both Figures III-2 and III-3.

Temperature conditioning of the flow model was accomplished with a water bath. A large metal tank was filled with water and the parallel plate assembly submerged in the tank. The tank had fill and drain provisions so that water could be circulated to provide a constant temperature. Water temperature was monitored by a type T thermocouple connected to a potentiometric temperature indicator. The flow of blood, conditioning media and wash solutions was controlled by a motor driven syringe. The motor driven syringe provided very precise flowrates through the system independent of system back pressure. A 50 ml syringe containing the fluid to be put through the flow model was mounted in the motor driven syringe and connected to the column with blood-compatible Tygon tubing. This tubing between the syringe and flow model entered the bottom of the water tank just below the flow model. A pinch valve on the tubing allowed the syringe to be refilled or replaced without backflow through the flow model. The flowing media was collected in test tubes or beakers from a Tygon tube leading from the top of the column.

## 2. Parallel Plate Model Test Results

A total of 15 flow tests were made with the model. A summary of test conditions is presented in Table IV. Heparinized whole blood was used in 13 tests (approximately 0.1 mg Heparin/ml). Buffy coat rich plasma was used for comparison purposes as the source of adhering cells in the other 2 tests. For each test, the column was pre-washed with a 20% heparinized plasma/Hank's Balanced Salt Solution (20% P/HBSS). The temperature was maintained within  $\pm 1^{\circ}\text{C}$  during each test. The test flowrate and plate spacing were selected based upon appropriate scaling for a full scale cell separator design. The objective in each of these tests was to characterize the distribution of the adhering cells on the glass plates as a function of the test conditions. Retrieval of adherent cells was investigated for test 18 only. For all other tests, the glass plates were removed from the flow network following cell separation, and the plates stained (Wright's stain) to permit a visual determination of the adherence process. General comments on each test are included in Table IV, where applicable.



TABLE IV SUMMARY OF FLOW TEST CONDITIONS WITH PARALLEL PLATE MODEL

TEST NO.	PLATE SPACING	FLOWRATE	TEMP. (+ 1°C)	QUANTITY OF WBC APPLIED	$\eta$ SEP (wbc)	$\eta$ SEP (adh)	TOTAL ADHERENT CELLS	COMMENTS
RNE 4	0.025 cm	0.764 ml/min	25°C	24 ml (buffy)	42%	60%	200x10 <sup>6</sup>	
RNE 5	0.025 cm	3.82 ml/min	37°C	47 ml	25%	37%	121x10 <sup>6</sup>	Most cells within 30 cm; cells grouped only within 5 cm.
RNE 6	0.025 cm	1.91 ml/min	37°C	18 ml (buffy)	44%		132x10 <sup>6</sup>	Groups of cells at entrance & 22 cm; smaller groups at 29 cm.
RNE 7	0.025 cm	0.764 ml/min	37°C	52 ml	20%	30%	88x10 <sup>6</sup>	
RNE 8	0.025 cm	1.91 ml/min	37°C	70 ml	32%	50%	196x10 <sup>6</sup>	Near saturation of column; cells forming monolayer.
RNE 9	0.025 cm	0.764 ml/min	37°C	100 ml	14%	49%	206x10 <sup>6</sup>	Dense covering of entire plates; large groups (100-500 cells)
RNE 10	0.013 cm	0.764 ml/min	37°C	68 ml	6%	10%	34x10 <sup>6</sup>	Considerable number of cells elongated and distended in shape.
RNE 11	0.013 cm	1.91 ml/min	37°C	56 ml	10%	15%	56x10 <sup>6</sup>	" " " "
RNE 12	0.020 cm	0.764 ml/min	25°C	51 ml	22%	32%	111x10 <sup>6</sup>	Dense cell population at entrance and beyond 25 cm from entrance.
RNE 13	0.020 cm	0.764 ml/min	37°C	61 ml	14%	27%	105x10 <sup>6</sup>	Dense cell population at entrance and beyond 25 cm.

TAVLE IV SUMMARY OF FLOW TEST CONDITIONS WITH PARALLEL PLATE MODEL (CONTINUED)

TEST	PLATE SPACING	FLOWRATE	TEMP. ( $\pm 1^{\circ}\text{C}$ )	QUANTITY OF WBC APPLIED	$\eta$ SEP (wbc)	$\eta$ SEP (adh)	TOTAL ADHERENT CELLS	COMMENTS
RNE 14	0.020 cm	1.91 ml/min	37°C	62 ml	12%	24%	91x10 <sup>6</sup>	Dense cell population at entrance and beyond 25 cm.
RNE 15	0.033 cm	0.764 ml/min	25°C	91 ml	19%	40%	119x10 <sup>6</sup>	Dense cell population at entrance and beyond 25 cm.
RNE 16	0.033 cm	0.764 ml/min	37°C	59 ml	22%	39%	106x10 <sup>6</sup>	
RNE 17	0.025 cm	0.764 ml/min	25°C	56 ml	13%	22%	57x10 <sup>6</sup>	
RNE 18	0.025 cm	0.764 ml/min	37°C	111 ml	18%	28%	188x10 <sup>6</sup>	Recovery of cells. $\eta_{\text{REC}} = 88\%$ .



Two distinct separation efficiencies are used in describing the performance of the process.  $\eta_{SEP(wbc)}$  represents the number of cells adhering to the plates as compared to the total number of white blood cells (adherent and nonadherent) applied to the column.

$\eta_{SEP(adh)}$  represents the percentage of cells attached to the plates as compared to the total number of adherent cells present in the original sample of whole blood. The adherent cell population includes monocytes as well as granulocytes. The number of cells adhering to the plates is calculated by subtracting the number of nonadherent cells present in the processed whole blood from the total number of cells in the original sample. Any cells 'lost' in the handling of blood, such as in syringes, beakers, tubing, etc., are thus counted as part of the adherent population. The separation efficiency values are therefore very conservative. The recovery efficiencies are likewise conservative in that the total cells in the eluate are compared to the total cells presumed adhering to the glass surfaces. No allowance is made for cells that may have stuck to the walls of containers.

Some general conclusions can be obtained from the data presented in Table IV. The use of buffy coat (Tests 4 and 6) rather than heparinized whole blood resulted in a greater percentage of adherent cells removed at the glass surface. The lower efficiencies shown for whole blood suggest that the presence of red cells in the flow field may interact to some extent in the migration and attachment of the adherent cells to the wall.

Calculations showed that a monolayer of cells covering the total surface area of the plates would contain approximately  $200 \times 10^6$  cells. (Assuming a leukocyte radius of 7 microns, the cell density in a monolayer would be  $6.5 \times 10^5$  cells/cm<sup>2</sup>.) This number of adherent cells was obtained during Tests 8 and 9. In Test 8, 70 ml of whole blood supplied the adherent cells whereas 100 ml was required during Test 9. However, the differential white blood counts of the two samples were considerably different, and the separation efficiency of the adherent cell populations were nearly identical, even at different operational flowrates.

Examination of the plates following testing indicated that the cells tended to group together. In the first 5 to 10 cm of column length, a dense uniform covering of the glass surface was observed. Beyond this point the cell density decreased as the distance from the entrance increased. Figures III-4 and III-5 show groupings of the adherent cells during Test 9 at 29 and 35 cm from the entrance end of the column. The direction of flow in these figures is from right to left.





Figure III-4 Grouping of Adherent Cells - Test 9  
29 cm from Column Entrance  
(Wright's Stain, Magnification. x40)

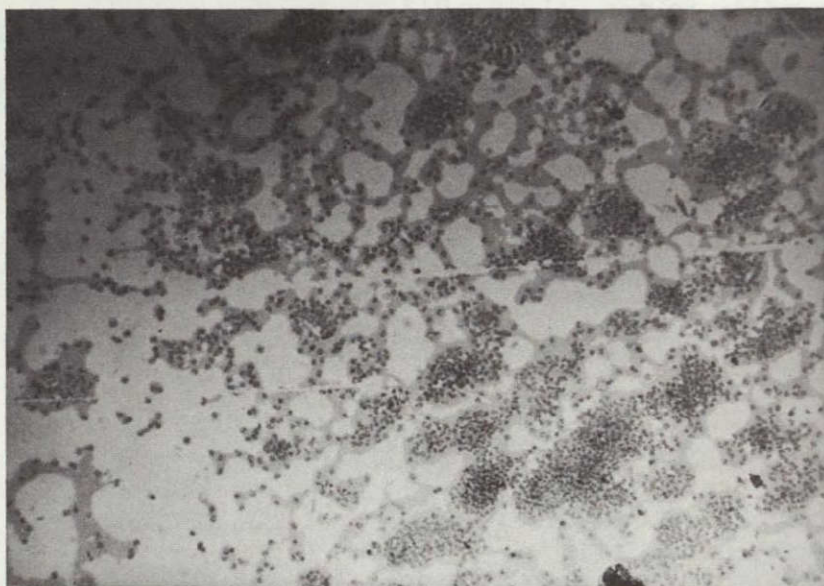


Figure III-5 Grouping of Adherent Cells - Test 9  
35 cm from Column Entrance  
(Wright's Stain, Magnification. x40)



A closer view of the cells in these groups is shown in Figures III-6 and III-7, respectively. Around each of the groupings a number of red cells and numerous platelets were observed. The presence of the nonadherent red cells was somewhat confusing until closer examination of the plates revealed that all cells were actually attached to an undercoating on the plate rather than directly to the glass surface. The adherence of cells to the undercoating is illustrated in Figure III-8. Apparently the plasma/HBSS preconditioning solution coats the surface, perhaps with proteins, to which the granulocytes and monocytes will selectively adhere with some slight contamination of red cells and platelets.

Another interesting visual observation was the respective locations and groupings of the granulocytes and monocytes. In general, for all tests, the granulocytes tended to appear throughout the length of the plates, being at the greatest concentration at the entrance end and decreasing in number by various amounts along the length of the plate, depending upon the flowrate and spacing. The monocytes were generally found clustered in close proximity with other monocytes, as shown in Figure III-9. At all flowrates tested, the majority of the monocytes were found between the middle of the column length and the exit of the flow section. In most cases, the eosinophils were also observed to be clustered together, as shown in Figure III-10. The eosinophils are indicated by the arrows. The clustering together of monocytes and eosinophils on the plates has not been explained.

A decrease in spacing between the plates from the initial 0.025 cm was investigated in tests 10 through 14. In tests 10 and 11, the spacing was 1/2 that of the previous 6 tests. Flowrates were comparable to those of earlier tests. A marked difference was observed in both the calculated total number of adherent cells and the physical appearance of the stained plates as observed microscopically. For these tests the cells did not tend to adhere in groups, as in tests with the wider flow annulus. In addition, the cells appeared to be elongated and distended in shape, as indicated by Figure III-11. The nuclei tended to stretch out into a long, thin shape. Also, considerable vacuolization of the adherent cells occurred, as shown in Figure III-12. The relatively thin spacing caused stagnation regions to form, thus disrupting the uniform flow across the width of the column. The surface area in contact with the flow was much reduced. This is partially responsible for the low number of adherent cells on the plates. In tests 12-14, the separation efficiency was somewhat higher, but the performance was still below that of the earlier tests with the 0.025 cm spacing.



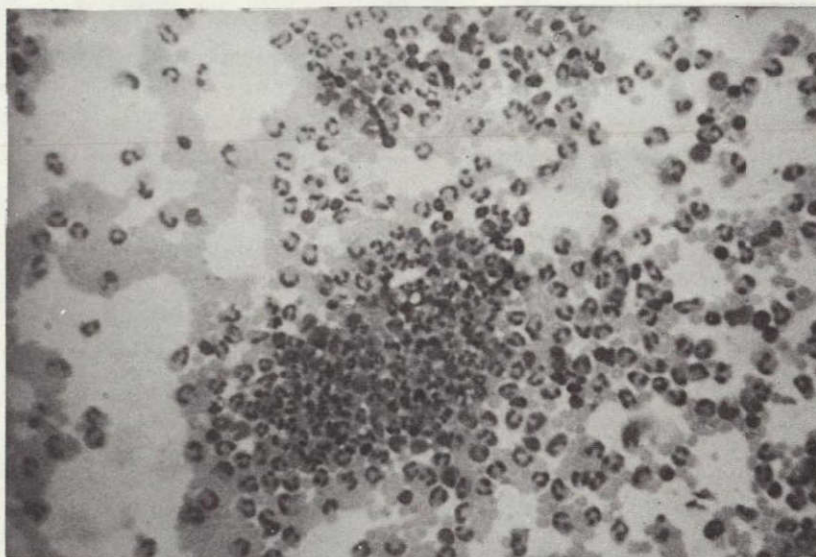


Figure III-6 Close-up of Cell Group - Test 9  
35 cm from Column Entrance  
(Wright's Stain, Magnification. x125)

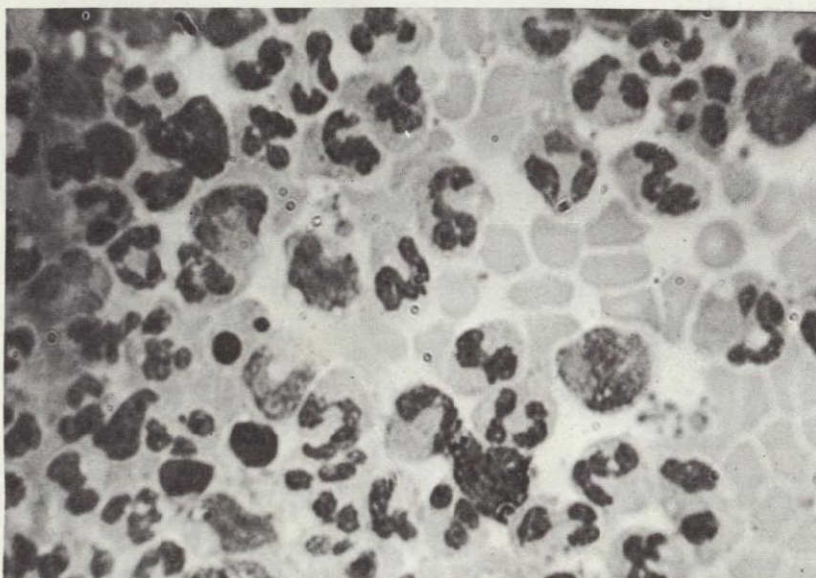
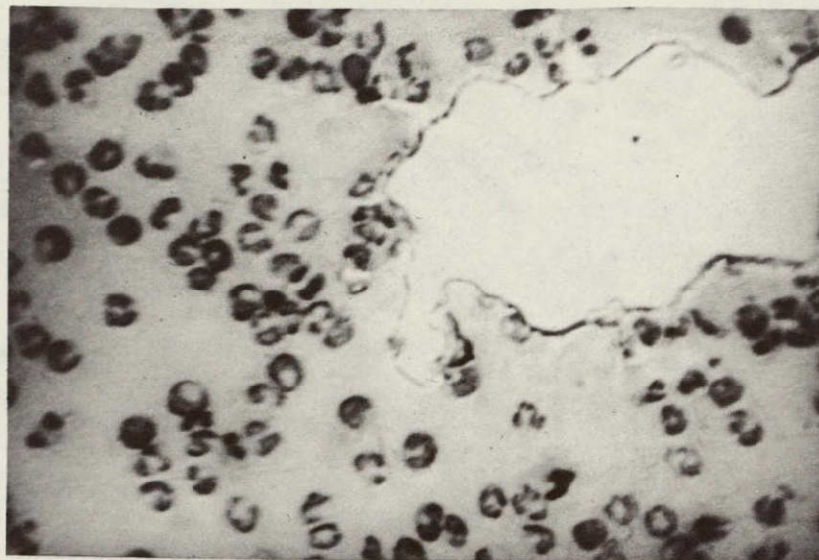
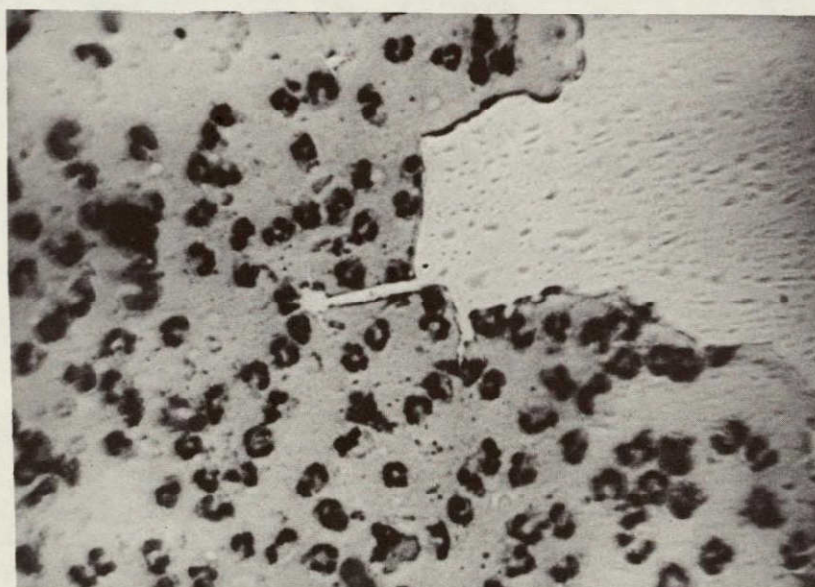


Figure III-7 Close-up of Cell Group - Test 9  
35 cm from Column Entrance  
(Wright's Stain, Magnification. x540)



A



B

Figure III-8 Undercoating on Glass to Which Cells  
are Attached - Test 8  
(Wright's Stain, Magnification. x250)



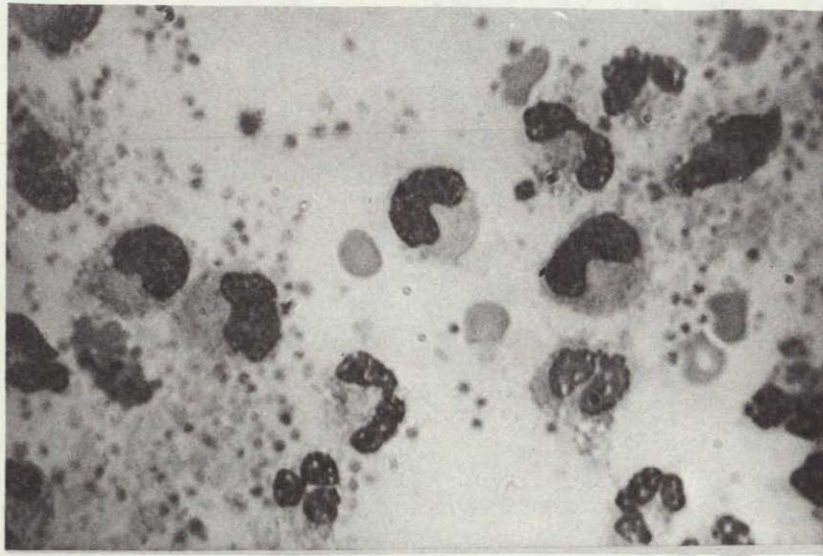


Figure III-9 Clustering of Monocytes - Test 9  
29 cm from Column Entrance  
(Wright's Stain, Magnification. x540)

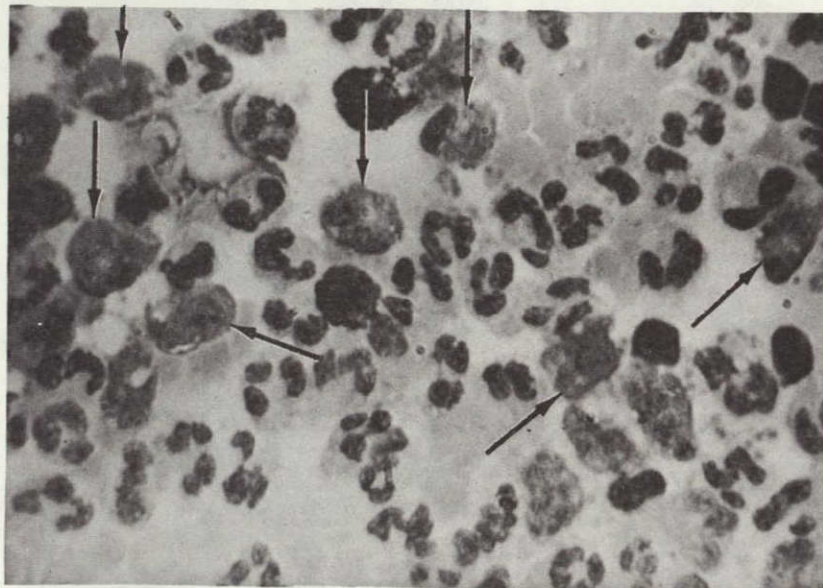


Figure -10 Clustering of Eosinophils - Test 9  
29 cm from Column Entrance  
(Wright's Stain, Magnification. x540)

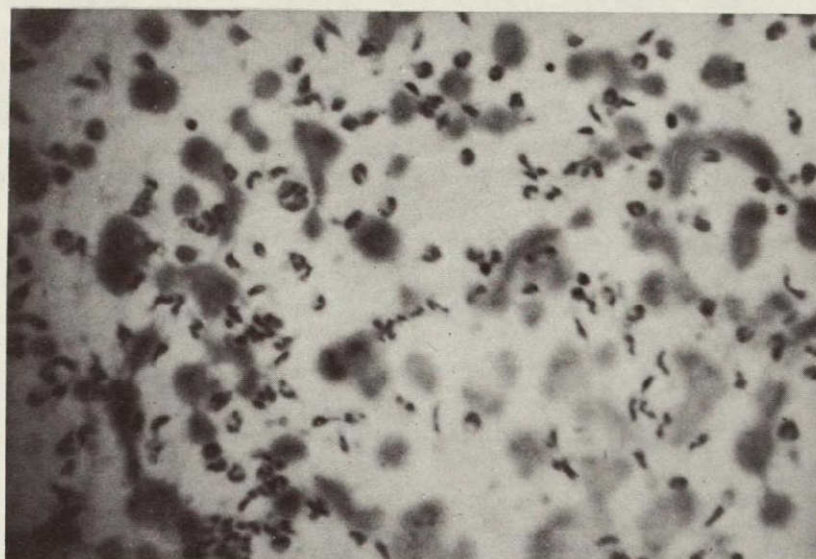


Figure III-11 Granulocytes Elongated and Distended  
in Shape - Test 10  
30 cm from Column Entrance  
(Wright's Stain, Magnification. x125)

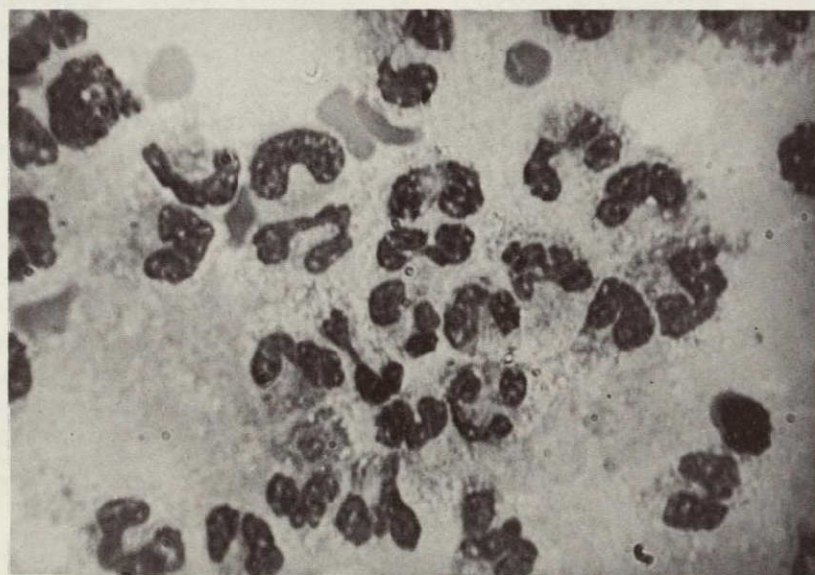


Figure III-12 Vacuolization in Adherent Cells - Test 10  
29 cm from Column Entrance  
(Wright's Stain, Magnification. x540)



Runs 15 and 16 were made with a larger spacing of 0.033 cm. The separation efficiency of the adherent cells was not as great as for runs 8 and 9. Visual examination of the adherent cell density along the plates indicated that many more cells were present beyond 25 cm than in the 10 to 15 cm region. At the flowrates tested, the spacing is great enough such that the time for adherent cells to migrate to the wall results in more cells present at a greater distance from the entrance to the columns. At this larger spacing, a longer column would be required to separate a given quantity of cells at a fixed flowrate; i.e., the entire surface near the entrance end of the column is not being saturated with cells during the separation process. The optimum spacing thus appears to be 0.025 cm.

The effect of dropping the temperature of the column during separation was given a cursory evaluation. Several tests were made with the column at room temperature (25°C). For several of the plate spacings tested there was no appreciable difference in separation efficiency within the accuracy of the measurements (Runs 12 and 13, and 15 and 16). However, for the preferred spacing of 0.025 cm, the separation efficiency dropped off considerably at the lower temperature (comparing Run 17 with Run 9).

Run 18 was made with the preferred spacing, flowrate and temperature to evaluate recovery of the adherent cells from the plates. The plates were nearly saturated with cells prior to flushing the column with an eluting solution for retrieval of the cells. The column was maintained at 37°C during the recovery process. The eluting solution was a mixture of 20% plasma and 5% 0.1M EDTA in Dulbecco's Phosphate Buffered Saline. This solution was evaluated during the Task B activity, as discussed in Chapter II. Eighty-eight (88) percent of the cells adhering to the column were recovered. Visual examination of the plates following recovery of cells indicated very few cells or cell debris remaining on the plates. Some cells may have been lost on glassware used for the collection and handling of cells. The eluting solution was run through the column at the same flowrate used for cell separation until approximately 11 ml of cell suspension was obtained. The solution present on the column was then allowed to incubate for one hour prior to further collection of cells. This sequence was repeated two more times. The summation of cells collected at each step in the process corresponds to the 88% efficiency value.



## B. CAPILLARY TUBE MODELS

Sufficient data were obtained using the parallel plate model to permit design and fabrication of a subscale test model using capillary glass tubes as the adhering surface. A plate spacing of 0.025 cm and length of 40 cm were selected as the preferred geometric parameters. It was desirable to use commercially available tubing due to cost and schedule constraints. However, several scaling considerations also influenced the selection of tube size and these are discussed below.

### 1. Design Considerations

The approach for the capillary tube separation column is to use both the inside and outside surfaces of very small diameter glass tubes for separating the adherent cells from whole blood. A section view showing the capillary tube geometry is presented in Figure III-13. In order to maintain the resistance of flow uniform across the cross section of the columns, the flow area across the capillaries,  $A_i$ , must closely approximate the flow area across the interstitial spaces,  $A^*$ . With equal flow volumes inside and outside the tubes, the inner and outer tube surfaces will be equally utilized in the separation and retrieval processes. It can be shown that the situation will exist when the outside diameter of the tubes is approximately four times the wall thickness.

Several relationships have also been derived for the flow volume per unit length (cross-sectional flow area) and the flow velocity through a column containing a large number,  $n$ , of capillary tubes. Flow volume is given by

$$\frac{(n-1)D^2}{2} \left[ \sqrt{3} - \frac{\pi}{2} \left( 1 - \frac{d^2}{D^2} \right) \right] \quad \left[ \frac{\text{cm}^3}{\text{cm}} \right]$$

where  $d$  is the inside diameter and  $D$  is the outside diameter of the tubes. The velocity of flow through the column is given by

$$\frac{\dot{V}}{\frac{nD^2}{2} \left[ \sqrt{3} - \frac{\pi}{2} \left( 1 - \frac{d^2}{D^2} \right) \right]} \quad \left[ \frac{\text{cm}}{\text{min}} \right]$$

where  $\dot{V}$  is the volumetric flowrate in  $\text{cm}^3/\text{min}$ .

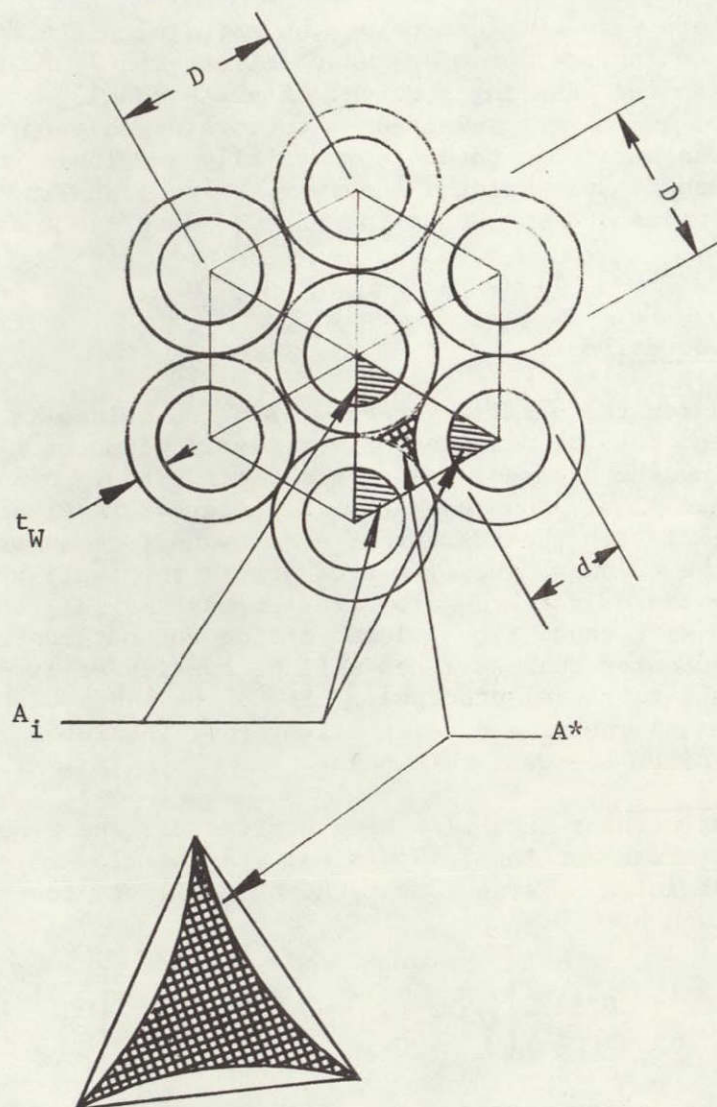


Figure III-13 Section View Showing Capillary Tube Geometry



Maintaining dynamic similarity between the optimum parallel plate model test conditions and the capillary tube model test conditions requires that the flow velocity through the tube matches that for flow between the parallel plates. A velocity of 11.7 cm/min through the parallel plate model yielded the best separation efficiency.

Two commercially available tube sizes were obtained that came closest to satisfying the design requirements. Nominal tube sizes were:

- 0.03 cm internal diameter (ID), 0.07 cm outside diameter (OD)
- 0.01 cm ID, 0.05 cm OD.

The tubes were PYREX glass (Dow Corning).

Testing columns containing tubes of these sizes (as discussed in paragraph 3 of this section) resulted in poor efficiency with the smaller diameter tubes and comparable performance with the parallel plate model results using the larger tube size. The possibility that an intermediate tube size might lead to improved separation and recovery efficiencies prompted a search for tubes having an internal diameter near 0.025 cm. Dow Corning agreed to make a limited production run of tubes 0.028 cm ID and 0.05 cm OD. Sufficient tubing was obtained to assemble several test columns. A description of the capillary tube column test hardware is presented below.

## 2. Capillary Tube Model Description

Glass columns normally used for chromatography were used as the containers for the capillary tubes. Two column sizes were used. The column subjected to the greatest amount of testing was 15 mm in diameter and 40 cm long. This column was used only with the 0.03 cm diameter tubes. Approximately 240 tubes could be packed into the column. Assuming a monolayer of cells forming on the inner and outer surfaces of the glass tubes, the column would hold approximately  $2 \times 10^9$  cells. The column contained a 100 mesh stainless steel screen at the entrance end to prevent tubes from slipping into the inlet port. An outer water jacket was used for temperature control. The 15 mm diameter column is shown in Figure III-14. Testing of the column is shown in Figure III-15.

Two different sets of end fittings were used with the 15 mm diameter column. In one set, designated as the baseline configuration, the flow into and away from the column was perpendicular to the flow through the capillary tube network. These end fittings are shown in Figure III-14. With the second set of fittings the flow enters and leaves the column along the column axis.



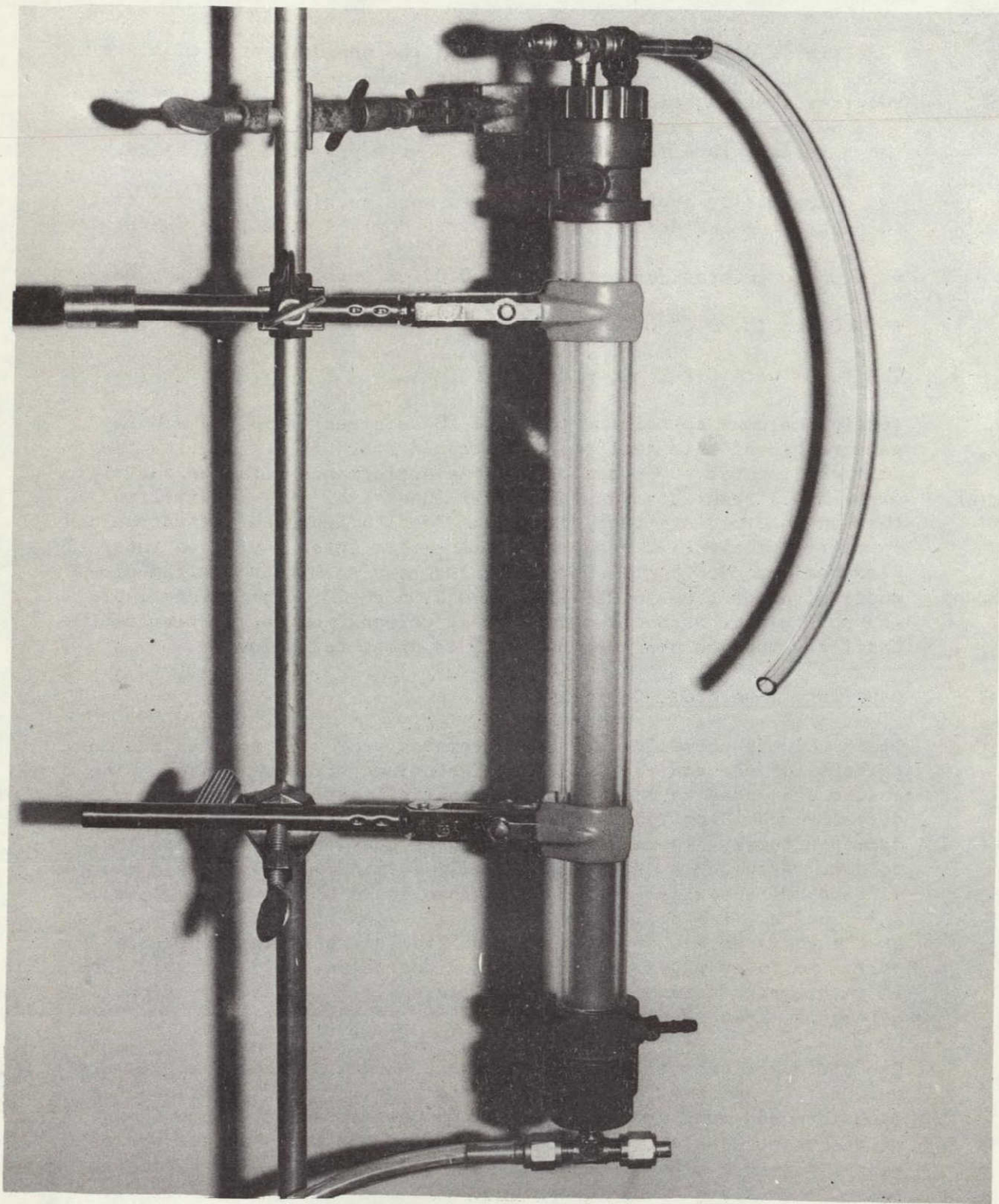


Figure III-14 Subscale Capillary Tube Separation Column



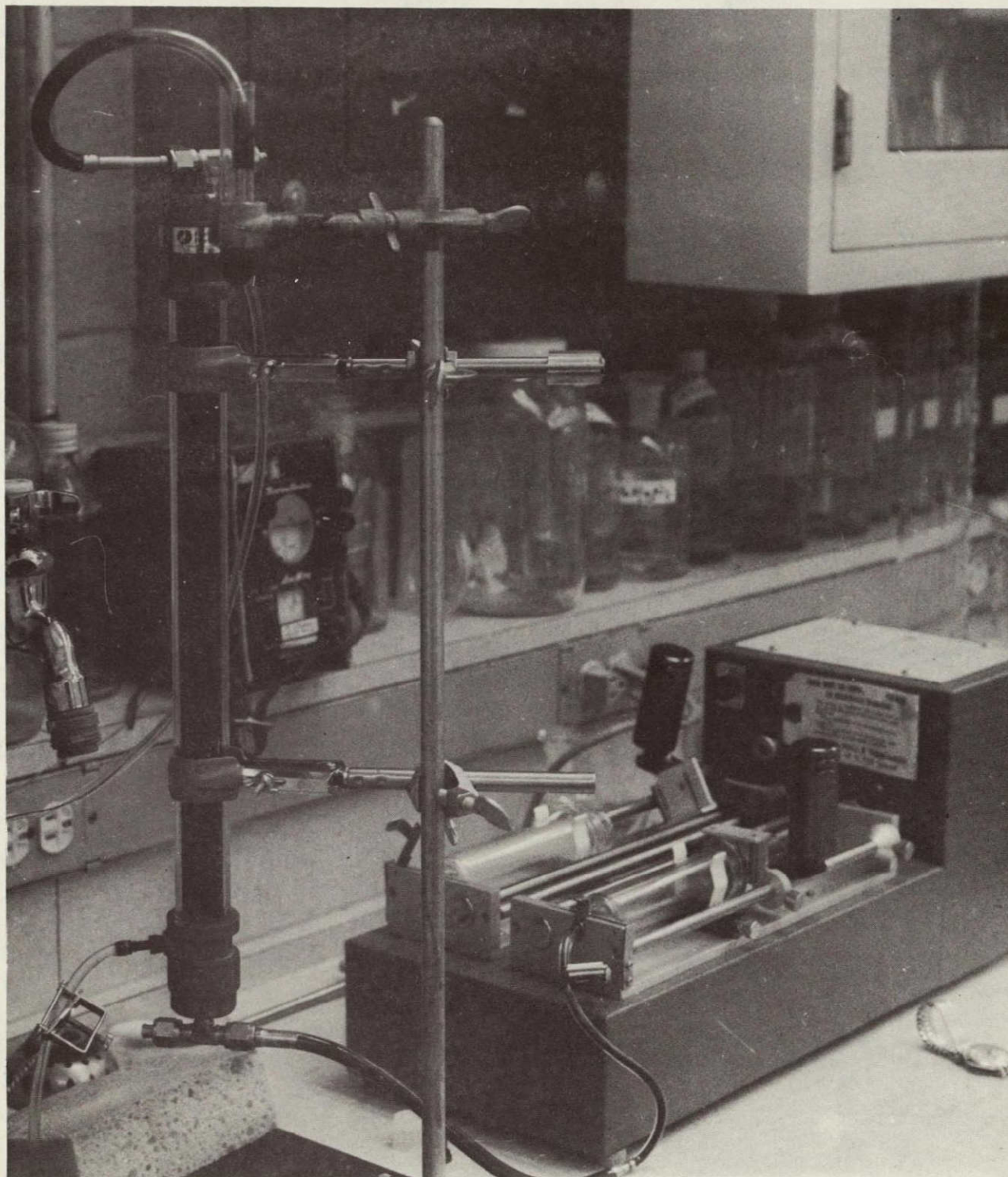


Figure III-15 Subscale Capillary Tube  
Separation Column in Test



A second column was assembled with the smaller diameter tubes. The column was 5 mm in diameter and held approximately 85 tubes. The length was also 40 cm, and the column contained an outer water jacket. The column would hold approximately  $3.5 \times 10^8$  cells in a monolayer when the 0.01 cm ID tubes were used or  $1 \times 10^9$  cells when the 0.028 cm ID tubes were used.

### 3. Capillary Tube Model Test Results

A summary of the pertinent test results obtained during capillary tube model testing is contained in Table V. A total of 19 separate tests were performed. Tests 24, 28, 29 and 36 were made with the 5 mm diameter columns. The remainder of the tests were made with columns 15 mm in overall diameter. The 20% plasma and 5% 0.1M EDTA in Dulbecco's PBS was used as the eluting solution for all tests through RNE 33. A 20% ACD (Formula A) homologous plasma in normal saline solution was used for Tests 34 and 35. The plasma was ABO compatible. ACD (Formula A) autologous plasma in normal saline was used as eluting solution for Tests 36 and 37.

The first test (Run 19) was made using the procedures, fluids, etc. established during testing with the parallel plate model. As mentioned previously, the flowrate was selected based on the desire to maintain the same velocity of blood flow through the capillary tube flow network as through the flow channel of the parallel plate model. At 7.64 ml/min the velocity was 10.9 cm/min. Eighty-nine (89) percent of the adherent cells were recovered from the column. This compares quite well with the 88% obtained using the parallel plate model (Test RNE 18, Table IV). Tests 20 through 23, and 25, were made to determine the sensitivity of the column to changes in blood flowrate, temperature and method of retrieving cells from the column. For Runs 20 through 23, the efficiency of separation of adherent cells from the whole blood was in general higher than for the parallel plate model. The low recovery efficiency for these tests resulted from attempts to wash the cells from the top of the column rather than the bottom as was the case for Test 19. In Test 25 the flowrate was increased by a factor of 2. Both the separation efficiency and recovery efficiency dropped considerably at this higher flowrate.

One test (Run 24) was made with the column containing the 0.01 cm ID tubes. For this configuration, the cross-sectional flow area outside the tubes was considerably greater than the flow area inside the tubes. A greater portion of the flow was between tubes. Thus full use of both the inside and outside surfaces of the tubes for adhering cells was not achieved. Also the recovery efficiency was decreased because the inside diameters of the tubes did not clear when the



TABLE V SUMMARY OF FLOW TESTING WITH CAPILLARY TUBE COLUMNS

TEST NO.	CAPILLARY TUBE SIZES	FLOWRATE	TEMP.	QUANTITY OF WBC APPLIED	$\eta$ SEP (wbc)	$\eta$ SEP (adh)	TOTAL ADHERENT CELLS	$\eta$ REC	COMMENTS
RNE 19	0.03 cm ID	7.64 ml/min	37°C	102 ml	36%	58%	196x10 <sup>6</sup>	89%	
RNE 20	" "	3.82 ml/min	37°C	104 ml	40%	52%	469x10 <sup>6</sup>	72%	
RNE 21	" "	7.64 ml/min	25°C	96 ml	26%	73%	172x10 <sup>6</sup>	63%	Highly lymphocytic; washed from top of column.
RNE 22	" "	7.64 ml/min	37°C	123 ml	38%	59%	330x10 <sup>6</sup>	65%	Washed from top of column.
RNE 23	" "	7.64 ml/min	37°C	280 ml	39%	59%	680x10 <sup>6</sup>	20%	Column not adequately washed.
RNE 24	0.01 cm ID	0.764 ml/min	37°C	130 ml	19%	32%	284x10 <sup>6</sup>	52%	ID of tubes did not clean with wash.
RNE 25	0.03 cm ID	15.3 ml/min	37°C	306 ml	10%	17%	214x10 <sup>6</sup>	29%	Flowrate too high.
RNE 26	" "	7.64 ml/min	37°C	257 ml	32%	43%	556x10 <sup>6</sup>	35%	Column at 4°C during wash.
RNE 27	" "	7.64 ml/min	37°C	170 ml	37%	55%	592x10 <sup>6</sup>	41%	Axial flow at inlet and outlet.
RNE 28	0.028 cm ID	3.82 ml/min	37°C	156 ml	19%	28%	321x10 <sup>6</sup>	18%	Screen clogging.
RNE 29	0.028 cm ID	1.91 ml/min	37°C	162 ml	32%	48%	512x10 <sup>6</sup>	10%	ID of tubes did not clear.
RNE 30	0.03 cm ID	7.64 ml/min	21°C	193 ml	39%	57%	1018x10 <sup>6</sup>	40%	Column washed at 21°C. Axial flow at inlet & outlet.

TAVLE V SUMMARY OF FLOW TESTING WITH CAPILLARY TUBE COLUMNS (CONTINUED)

TEST NO.	CAPILLARY TUBE SIZES	FLOWRATE	TEMP.	QUANTY OF WBC APPLIED	$\eta$ SEP (wbc)	$\eta$ SEP (adh)	TOTAL ADHERENT CELLS	$\eta$ REC	COMMENTS
RNE 31	0.03 cm ID	7.64 ml/min	37°C	122 ml	49%	73%	467x10 <sup>6</sup>	44%	Column washed from top.
RNE 32	" "	7.64 ml/min	37°C	130 ml	17%	26%	s33x10 <sup>6</sup>	29%	Increased flow rate of eluting solution.
RNE 33	" "	7.64 ml/min	37°C	168 ml	19%	29%	431x10 <sup>6</sup>	72%	Greater quantity of eluting solution applied.
RNE 34	" "	7.64 ml/min	37°C	289 ml	36%	66%	776x10 <sup>6</sup>	61%	20% ABO compatible Homologous plasma in normal saline eluting solution.
RNE 35	" "	7.64 ml/min	20°C	306 ml	22%	41%	532x10 <sup>6</sup>	25%	Wash at 20°C.
RNE 36	0.028 cm ID	1.91 ml/min	37°C	132 ml	11%	16%	226x10 <sup>6</sup>	63%	ACD in normal saline eluting solution.
RNE 37	0.030 cm ID	7.64 ml/min	37°C	213 ml	24%	51%	452x10 <sup>6</sup>	61%	ACD in normal saline eluting solution.



wash solution was added to the column. It appeared from this test that the tube size was too far from the optimum value established during the parallel plate testing.

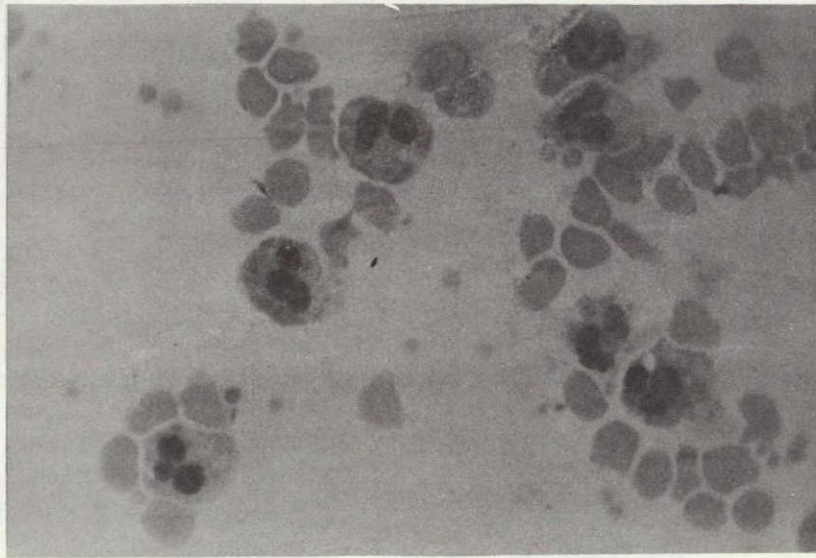
Test 26 evaluated the effect of dropping the temperature to approximately 4°C at the time the wash solution was introduced into the column. The same wash solution used in previous testing was incubated in the column at 4°C. The eluting solution was extracted from the column at 1-hour intervals and after 2 hours only 22% of the adherent cells had been recovered from the column. The temperature was then increased to 37°C and after an additional hour another cell count was made. An additional 13% of the adherent cells were recovered. The total quantity of cells recovered was 35% of the total number adhering to the tubes. This compares to recoveries of 60% or greater when all eluting of the cells from the surface was accomplished at 37°C.

Modifications to the inlet and outlet regions of the column were investigated in Tests 27 and 30. As mentioned in the previous section, the baseline capillary tube column was configured with the inlet and outlet tubes directing flow at the entrance and exit to the column perpendicular to flow through the capillary tube network. When the flow was introduced to the column parallel to the axis of the capillary tubes, comparable separation efficiency but reduced recovery efficiency was obtained. Perhaps when eluting solution is introduced perpendicular to the tube axes some turbulence is generated which aids in the flushing of cells from the tube surfaces.

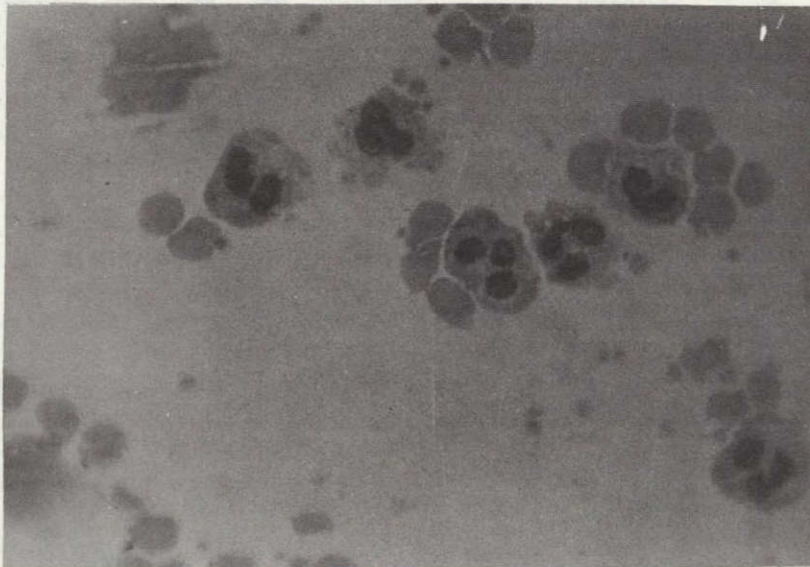
Although the recovery efficiency for test 30 was somewhat decreased, the total number of adherent cells collected from the original sample was quite high due to a slightly elevated white blood cell count.  $4 \times 10^8$  granulocytes and monocytes were recovered from 193 ml of whole blood. Since the cells recovered during this test are typical of cells retrieved throughout the capillary tube column test activity, some examples are shown in Figures III-16 through III-19. A few residual red cells were flushed from the column with the first few column volumes of wash solution. In subsequent wash fractions, when the monocytes began to appear, considerable numbers of platelets were also collected, as indicated by Figure III-18. Some monocytes (and granulocytes) obtained in the latter wash fractions showed some vasculization, as indicated by the arrows in Figure III-19.

Back-flushing the column by flowing the wash solution in a direction opposite to that of the whole blood during separation also resulted in a reduced recovery efficiency, as seen by the data for Test 31. However, the mononuclear cells showed less vasculization, as





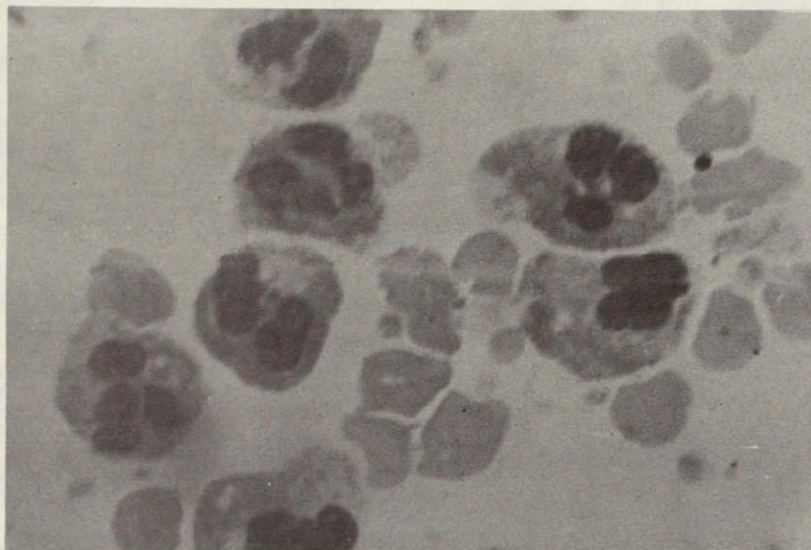
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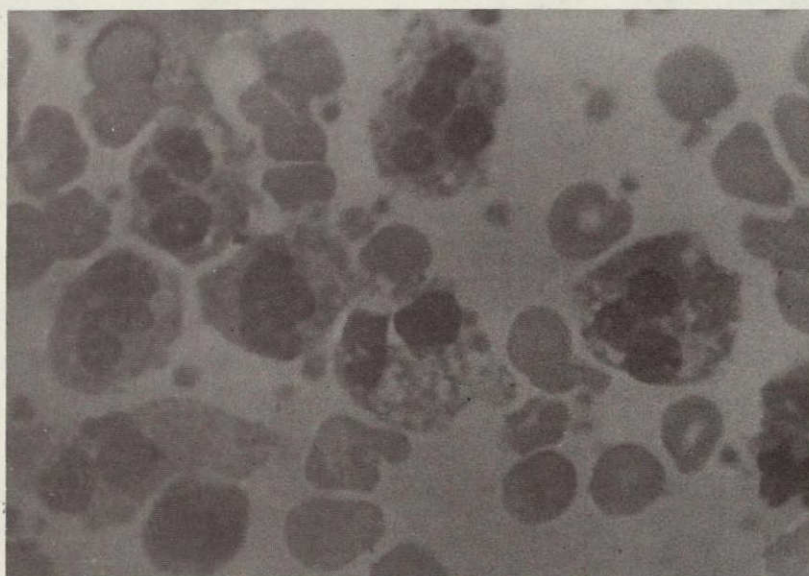
B

Figure III-16 Granulocytes Collected Using EDTA  
Eluting Solution - Test 30  
(Wright's Stain, Magnification. x540)





A



B

Figure III-17 Close-up of Granulocytes Collected  
Using EDTA Eluting Solution  
(Wright's Stain, Magnification. x1000)

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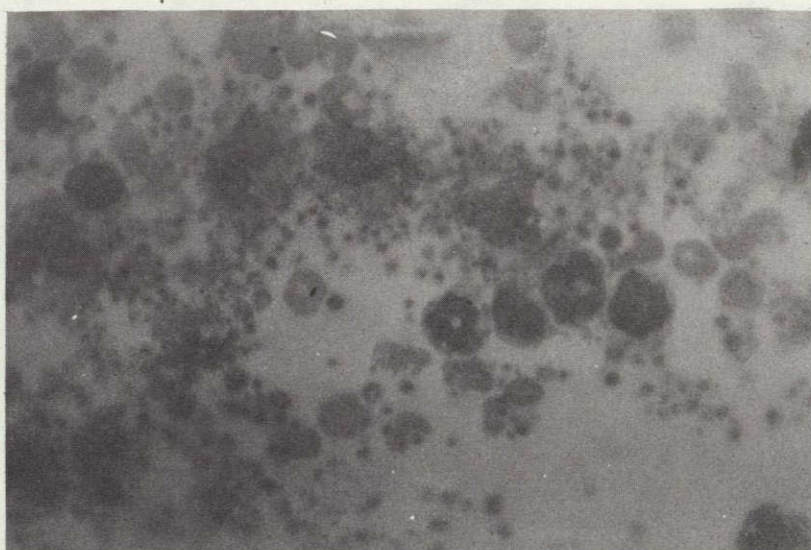


Figure III-18 Monocytes Collected Using EDTA  
Eluting Solution - Test 30  
(Wright Stain, Magnification. x540)

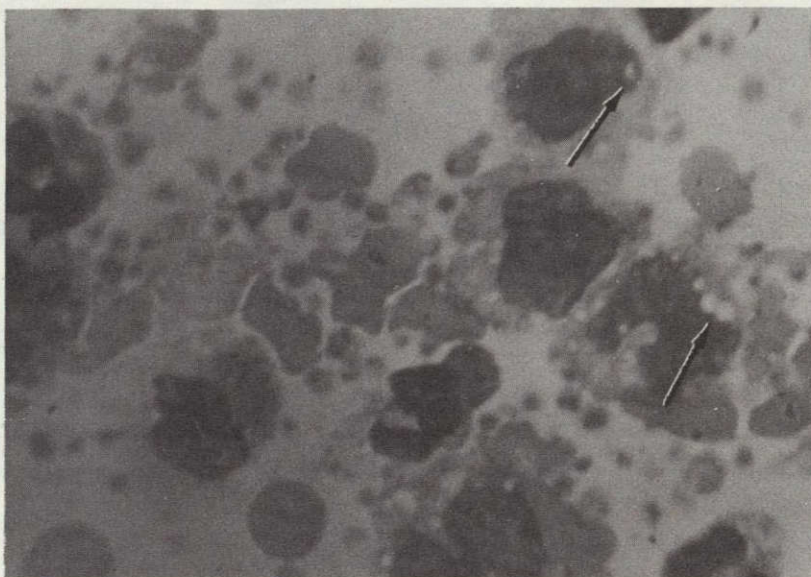


Figure III-19 Close-up of Monocytes Collected  
Using EDTA Eluting Solution - Test 30  
(Wright's Stain, Magnification. x1000)



illustrated by Figures III-20 and III-21. Apparently the cell fractions which were collected following the inflow of considerable eluting solution in Test 30 failed to release during washing of the column in this test. The cells obtained rather easily in the elution process contained little vacuolization. It is unclear whether the reduced efficiency during back-flushing resulted from the death of cells or the inability to detach the cells from the wall.

Tests 28, 29 and 36 were made with the 0.028 cm internal diameter tubes. In test 28, clogging of the column at the entrance end was observed at the conclusion of the test. This restricted the cross-sectional flow area and made inoperative a considerable portion of the attaching surface area. This accounts for the reduced separation efficiency. For test 29, the screen retaining the tubes at the entrance end of the column was removed and the separation efficiency was comparable to that obtained in other tests. However, the recovery efficiency was extremely low. The inside volumes of the tubes did not clear of red cells, even after repeated flushing at extremely high flowrates. Obviously, the inside surfaces of the tubes were not being properly used for separation, and once filled with blood, clogging prevented retrieval of the cells. This data indicated that the flow field of whole blood through the small diameter tube is markedly different than through a gap of the same dimension between flat surfaces.

For Test run 36, the test conditions were selected in an attempt to improve both phases of the process. The flowrate of whole blood through the column was reduced to enhance the separation efficiency, and the flowrate and quantity of eluting solution were both increased to enhance the recovery of cells from the tube surfaces. The separation efficiency was not significantly different than in previous testing. The recovery efficiency increased to a level typical of the average recovery of cells using the larger diameter tubes. When the yield of cells collected from the whole blood is considered, however, the low separation efficiency produces a low total process efficiency. This is true for all testing with the 0.028 cm tubes. The inconsistency of results with these tubes suggested that the 0.030 cm diameter tubes represented the preferred geometry.

Test run 35 was made to determine if performing the separation at room temperature would yield comparable results to that where the column was maintained at 37°C with an external heat source. Testing was performed with the column at 20°C. Both the separation and recovery efficiencies were reduced. Previous testing (Test run 21) at 25°C had yielded total collection of cells comparable to that at 37°C. The threshold temperature for decreased adhesiveness and subsequent detachment of cells from the wall is thus in the range of 20 to 25°C.



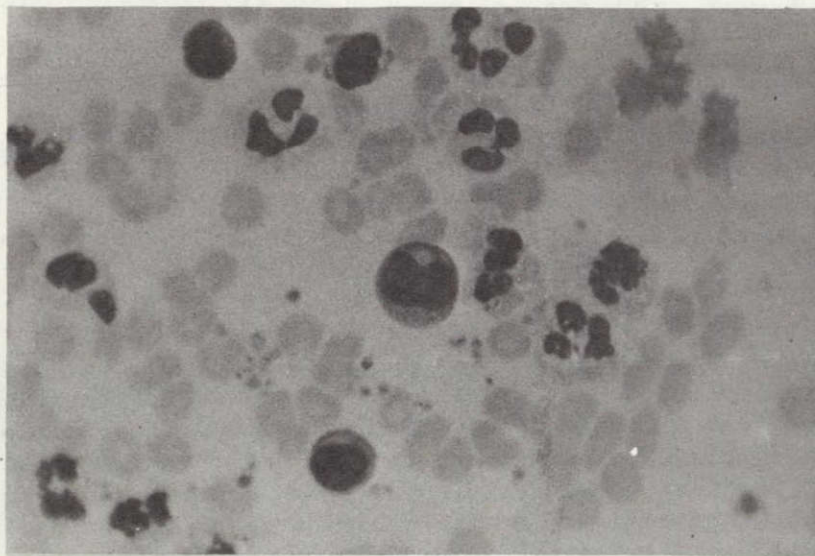


Figure III-20 Cells Collected Using Edta Eluting  
Solution - Test 31  
(Geimsa Stain, Magnification. x540)

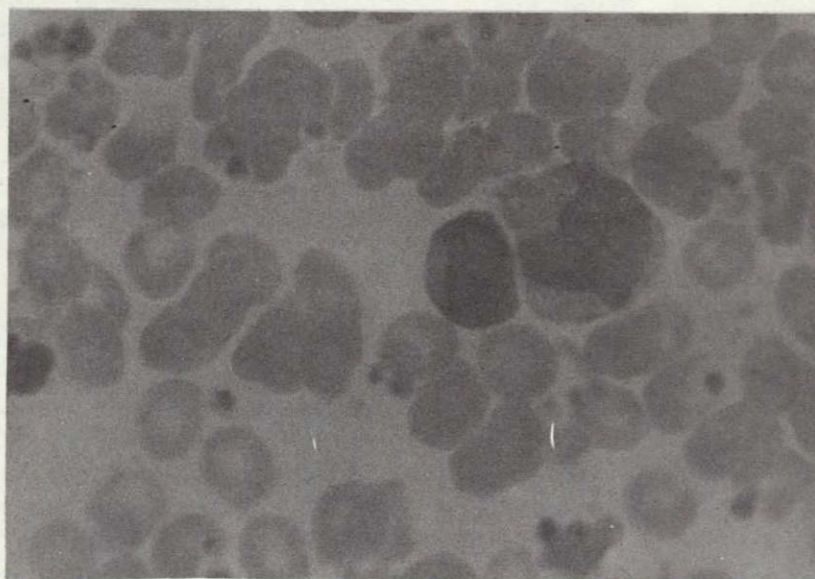


Figure III-21 Monocytes Collected Using EDTA  
Eluting Solution - Test 31  
(Geimsa Stain, Magnification. x1000)



Several different eluting solutions were evaluated for Tests 34 through 37. In run 34, ABO compatible homologous plasma (collected in ACD, Formula A) was mixed in a ratio of 1:5 with normal saline. The recovery efficiency was comparable to the average value obtained for the baseline configuration using the EDTA solutions. (It should be noted that the recovery efficiency for run 19 represents the highest yield obtained in all testing. The average recovery for the baseline geometry and preferred operating conditions falls in the range of 60 to 70%.) An example of the cells collected during Test 34 is shown in Figure III-21. A close-up of the cells, showing several monocytes, is included as Figure III-23.

ACD was mixed with normal saline and heparinized plasma from the donor and used as the eluting solution for test run 37. The results are comparable to those obtained for run 34 and the earlier testing with EDTA. Since ACD is preferred to EDTA as the fluid media for containing the collected cells for reinfusion into the donor, and since the recovery efficiency is comparable with the two fluids (within the accuracy of the test), ACD becomes the eluting solution of choice.

The viability of monocytes from test run 34 was evaluated by using the cells as feeder layers in the cell culture system. Samples of cells collected at various intervals during the eluting process were plated in Agar-gel and covered with separated human bone marrow. The bone marrow overlay contained non-glass adherent mononuclear cells. As expected, the first several fractions of cells, which contained very few monocytes, produces few colonies of cells in the culture plates. Fraction 3, however, yielded about 80% of the number of colonies present when normal feeder layers were used. Normal feeders were prepared by sedimenting whole blood, and drawing off the buffy coat, which was then counted and used as control in the assay. In all cases  $1 \times 10^6$  cells were plated as feeder layers against 50,000 separated human marrow cells. The ability to stimulate cell growth provides an indication of the monocyte viability and functional capability. The only conclusion that can be drawn regarding granulocyte viability from this assay is that the presence of granulocytes in the feeder layers did not inhibit the capability of the monocytes to produce colony growth.



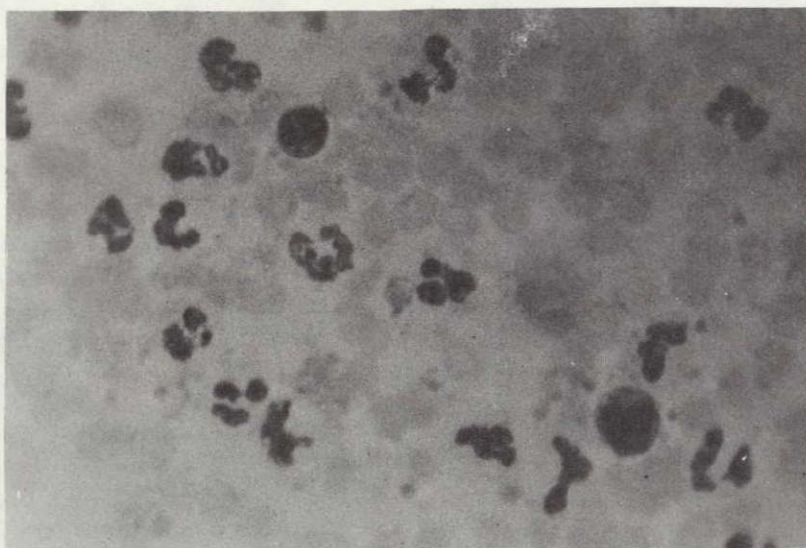


Figure III-22 Cells Collected Using Homologous ACD  
(Formula A) Plasma/Saline - Test 34  
(Geimsa Stain, Magnification. x 540)

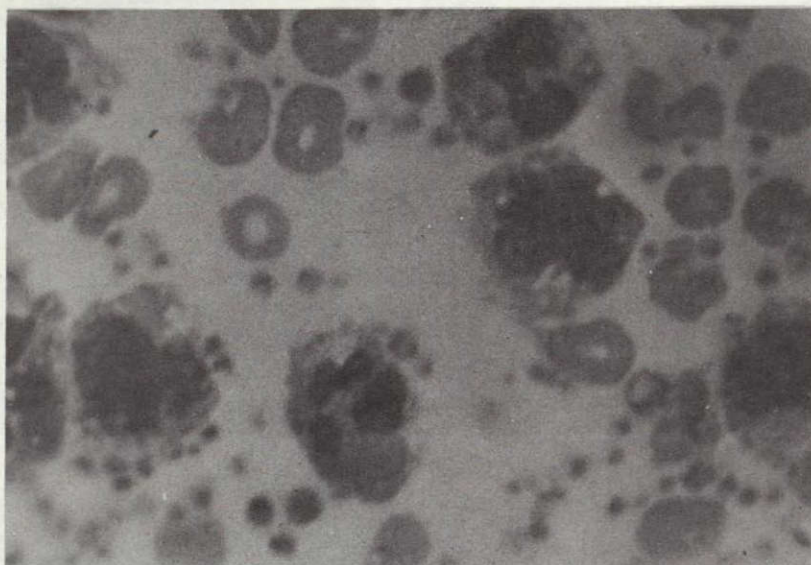


Figure III-23 Close-up of Cells Collected Using  
Homologous ACD (Formula A)  
Plasma/Saline - Test 34  
(Geimsa Stain, Magnification. x 1000)

model. Recovery efficiencies as high as 89% of the adhering cell population were obtained. A velocity of whole blood through the tubes of 10.9 cm/min was selected as preferred for cell separation. Both the separation and retrieval portions of the process appeared to be optimized at 37°C. Lowering the temperature below 20°C during the elution process resulted in decreased recovery efficiency.

A 20% ACD (Formula A) plasma/normal saline mixture was evaluated as an eluting solution with the capillary tube columns and was found to be as effective as the two solutions mentioned above for recovering cells from the column. Since ACD is preferred to EDTA for clinical usages, the ACD plasma/saline eluting solution is the one of choice. Limited in-vitro testing showed that greater than 90% of the eluted granulocytes would phagocytize latex particles even though morphologically some of the recovered cells contained vacuoles and plasma membrane surface irregularities. Monocytes recovered from the capillary tube column using the ACD (Formula A) plasma/saline solution were found to retain their capability to stimulate human bone marrow growth in cell culture.

We recommend that a Phase II effort be initiated involving the analysis, design, fabrication and test of a prototype separator. Basic concept feasibility of the technique has been demonstrated. It is recommended that we proceed to the design, fabrication and testing of a prototype device that can be operated on a continuous basis to recover large quantities of granulocytes and monocytes from a single donor. Test verification of the prototype separator can be accomplished by operating the device in a continuous-flow manner at a predetermined flowrate in order to provide sufficient adherent cells to saturate the column. The use of dogs as leukopheresis donors would permit this type of evaluation with the prototype device. Specifically, the effects of the device and associated procedures on the donor, and the viability of the processed blood components could be determined. A program plan outlining the Phase II effort is contained in Reference 22.

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#### APPENDIX - COLONY GROWTH OF HUMAN BONE MARROW CELLS IN-VITRO

A technique for growing human bone marrow cell colonies in agar-gel medium is described in this section. The technique was developed by Dr. William A. Robinson at the University of Colorado Medical Center in Denver, Colorado. Additional details describing the basic procedure can be found in References 19 and 20.

"Feeder Layers" containing  $1 \times 10^6$  normal human peripheral white blood cells are used as the stimulus for colony growth. Either an unseparated mixture of leukocytes from peripheral blood, or glass adherent mononuclear cells, when plated as overlays, will produce the colony growth. For underlays, there should not be more than one red blood cell for each white blood cell plated. Human bone marrow aspirates are collected in heparinized syringes and plated as  $2 \times 10^5$  cells in an overlay on the "feeder layers". Normal human bone marrow yields 32-102 colonies per  $2 \times 10^5$  cells plated. Colonies appear to be highly granulocytic. Colonies reach a size of 500-1500 cells at 12-16 days. Increased numbers of colonies can be obtained if the bone marrow is separated by one of the gradient separation techniques and 50,000 non-glass adherent mononuclear cells are used as the overlay.

A modified preparation of McCoy's 5A medium (Biogen Laboratories, Denver, Colorado) to which has been added 15% fetal calf serum and a variety of other nutrient elements is used as the medium to prepare both underlays and overlays. Human serum is particularly avoided since it contains factors which alter colony growth in this system. McCoy's 5A medium is mixed in a 9:1 concentration with boiled 5% agar (Difco-Bacto-Agar) to give a final agar concentration of 0.5%. The medium must be warmed to 37°C prior to adding the agar to prevent gelling. The agar is added to the medium at a temperature just below the boiling point. The mixture is then allowed to cool to approximately 40°C. To this mixture is then added the peripheral white blood cells in plasma to the desired concentration. One milliliter aliquots of this are then pipetted into 35 mm plastic petri dishes (Falcon Plastics, Los Angeles, California). The agar medium is then allowed to gel at room temperature. Overlays may be pipetted directly onto the underlays once they have gelled or the plates may be stored at 37°C in a humidified incubator for up to 14 days without loss of colony stimulating activity.

All human bone marrow for culture is obtained from the posterior iliac crest using a Westerman-Jensen needle. The specimen for culture is collected by aspirating 5-10 ml of bone marrow into a syringe

rinsed with 1:1000 heparin just prior to use, with care taken not to leave any quantity of heparin in the syringe. The aspirate is then transferred to a test tube. The plasma containing white blood cells, platelets and small fat particles is removed with a Pasteur pipette. This is sedimented in a bench centrifuge and washed three times in McCoy's 5A medium. No attempt is made to break apart the fat particles, as these are removed during the washing procedure. McCoy's 5A medium is then mixed in a 9:1 concentration with boiled 3% agar. Washed bone marrow cells are added to this mixture in a concentration of  $2 \times 10^5$  cells/ml and plated on top of the previously prepared underlays. After gelling at room temperature, the plates are incubated at 37°C in a humidified incubator with a constant flow of 7.5% CO<sub>2</sub> in air.

Colonies are visible with a dissecting microscope at 4-5 days of incubation. Colonies increase in size from day 6 to 10. A rapid increase in size occurs to days 15 to 20, when maximum colony size is reached.